INTERACTIONS OF DIPLOID EMBRYONAL CARCINOMA CELLS AND NORMAL INNER CELL MASS CELLS WITH 2.5 DAY MOUSE EMBRYOS FOLLOWING AGGREGATION

by

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ABSTRACT

Spontaneous teratocarcinomas are ovarian or testicular tumors which have their origins in germ cells. The tumors contain a disorganized array of benign differentiated cells as well as an undifferentiated population of malignant stem cells, the embryonal carcinoma or EC cells. These pluripotent stem cells in tissue culture share many properties with the transient pluripotent cells of the early embryo, and might therefore serve as models for the investigation of developmental events in vitro.

The property of EC cells of prime interest in this study is an in vivo phenomenon. Certain EC cell lines are known to be regulated in vivo and to differentiate normally in association with normal embryonic cells, resulting in chimeric mice. These mice have two genetically distinct cell populations, one of which is derived from the originally malignant EC cells. This has usually been accomplished by injection of the EC cells into the Day 3 blastocyst. In this study, the interactions between earlier stage embryos and EC cells have been tested by aggregating clumps of EC cells with Day 2 embryos. The few previous aggregation studies produced a high degree of abnormality in chimeric embryos, but the EC cells employed had known chromosomal abnormalities. In this study, two diploid EC cell lines (P19 and P10) were aggregated with 2.5 day mouse embryos, and were found to behave quite differently in the embryonic environment. P19 containing aggregates generally resorbed early, and the few embryos recovered at midgestation were normal and non-chimeric. P10 containing aggregates survived in high numbers to midgestation, and the P10 cells were very successful in colonizing the embryo. All these embryos were chimeric, and the

contribution by the EC cells to each chimera was very high. However, these heavily chimeric embryos were all abnormal.

Blastocyst injection had previously produced some abnormal embryos with high P10 contributions in addition to the live born mice, which had lower EC contributions. This study now adds more support to the hypothesis that high EC contributions may be incompatible with normal development. The possibility that the abnormalities were due to the mixing of temporally asynchronous embryonic cell types in the aggregates was tested by aggregating normal pluripotent cells taken from 3.5 day embryos with 2.5 day embryos. Early embryo loss was very high, and histological studies showed that the majority of these embryos died by 6.5 days development. Some embryos escaped this early death such that some healthy chimeras were recovered, in contrast to recovery of abnormal chimeric embryos following P10-morula aggregations, and non-chimeric embryos following P19-morula aggregations.

This somewhat surprising adverse effect on development following aggregation of normal cell types suggests that there are developmental difficulties associated with the mixing of asynchronous cell types in aggregates. However, the greater magnitude of the adverse effects when the aggregates contained tumor derived cells suggests that EC cells should not be considered the complete equivalent of the pluripotent cells of the early embryo.

INTRODUCTION

THE QUESTIONS RAISED IN THIS STUDY

The phenomenon of regulation and participation in normal development by cultured EC cells upon their return to the embryonic envirnonment is of particular interest in this study. The P10 and P19 cell lines seemed to be ideal candidates as EC cell lines which could be used to explore more completely the effects of aggregating EC cells with morulae. They are euploid and their response to the embryonic environment following blastocyst injection has been fully explored. It was hoped that by aggregating these cells with early morulae chimeras might be generated at an even higher frequency and that the EC cells would predominate in at least some of the chimeras such that the in vivo developmental potential of euploid EC cells could be more completely assessed.

The following questions are considered in this study.

1. Aggregation is an efficient technique for producing EC-embryo chimeras, but apparently produces a high level of abnormality. Is there really a difference in the rate of abnormal development produced by aggregation compared to blastocyst injection?

2. If so, does a high rate of abnormality correlate with higher EC contributions achieved in aggregation chimeras?

3. Alternatively, are there developmental problems associated with mismatching of cells from different developmental stages? If so, ICM-morula aggregates would also be expected to show abnormalities of development.

4. Can an improvement in the developmental performance of an EC cell line be induced by a pretreatment of EC cells with retinoic acid? If so, P19 cells, which seem to persist to some degree as stem cells in all chimeras, might be committed to differentiation and more normal development as a result of retinoic acid pretreatment.

LITERATURE SURVEY

EARLY DEVELOPMENT OF MOUSE EMBRYOS

A preliminary overview

One very broad division of mammalian development is into pre-implantation and post-implantation stages. In the pre-implantation stage, the free living embryo is most accessible to experimental manipulation, whereas following implantation such manipulations become very much more difficult. Fortunately, critical developmental events take place before implantation and the study of these events is pertinent to central issues of cellular differentiation and development.

In this study, two pre-implantation stages of development are of particular interest: the 2.5 day morula and the 3.5 day blastocyst. It will be useful to begin, therefore, with an overview of the early development of mouse embryos, in order to define these two pre-implantation embryo stages, and to describe the events preceding and immediately following their formation.

In their 1966 description of the early embryology of the mouse, Snell and Stevens present a comprehensive review of pre-implantation development which has been used extensively in the preparation of this overview.

A female in estrus will ovulate as many as 15 to 20 eggs. Each egg is enclosed within a transparent zona pellucida, a mucoprotein produced by the surrounding ovarian follicular cells. The zonae must be removed from pre-implantation embryos prior to experimental manipulations, and Mintz (1962) noted that embryos become "sticky" following liberation from the zonae. She suggested that in addition to one proposed function of the zona, which is to assist retention of the relatively loosely associated cells during the early

cleavage divisions, the zonae might serve to prevent natural aggregation of cleavage stage embryos.

A female in estrus becomes receptive to mating about 2 hours prior to ovulation such that sperm are present in the upper reaches of the oviducts at the time of ovulation, and fertilization occurs almost immediately. However, the first cleavage division does not occur until about 24 hours later. Mating occurs during the dark cycle, and due to the delay before first cleavage, single cell embryos can be recovered on the first morning after mating. By a widely accepted, though not universal convention, the day on which the vaginal plug is found is referred to as Day 0.5 of mouse pregnancy.

On Day 1 of pregnancy, 2-cell embryos can be recovered from the oviducts, and the pace of the cleavage divisions then increases, with the divisions remaining nearly synchronous. By early morning of Day 2 of pregnancy, 8-cell embryos can be recovered from the oviducts. Because cleavage involves cell divisions without growth, this embryo is the same size as the egg. The process of compaction begins at the 8-cell stage. During compaction, the cell outlines are lost as the cells flatten against one another and establish cell-cell communications via gap junction formation (Goodall and Johnson, 1984). From this stage onwards the embryo is known as a morula. At about the 32-cell stage, a cavity begins to form within the morula. This cavity expands rapidly, such that by Day 3 of pregnancy, the hollow, fluid-filled structure known as the blastocyst has developed. The blastocyst represents a landmark stage in development because the first differentiation of cell types occurs during its formation. The blastocyst consists of two cell types: firstly, a single layer of cells known as the trophectoderm surrounding the blastocoelic cavity. Some of the cells of the trophectoderm will go on to form the fetal portion of the placenta (Gardner, et al., 1973). Secondly, within this cavity, at one pole,

there is a small cluster of cells known as the inner cell mass (ICM). At the 64-cell stage, the ICM consists of about 15 cells, some of which are destined to form the extraembryonic membranes and a few, perhaps as few as three cells in the early blastocyst (Markert and Petters, 1978) are destined to become the embryo proper.

During the early cleavage divisions the embryos move through the oviducts, and at about the time of cavitation, the morulae enter the uterus. Blastocyst formation then occurs within the uterus. The blastocysts are still enclosed within their zonae, and are still the size of the egg. By Day 4 of pregnancy, the mature blastocysts hatch from their zonae and implant within the bicornuate uterus. The blastocysts implant in uterine crypts and the uterine epithelium in the region degenerates and is sloughed off. At the same time, there is a proliferation of endometrial stromal cells giving rise to the maternal deciduum (reviewed by Kearns and Lala, 1983) such that by Day 5, a visible swelling is present in the uterus at each implantation site. The pre-implantation development of mouse embryos is summarized diagrammatically in Figure 1.

Rapid growth of the embryo begins with implantation. Further cell differentiations occur by the late blastocyst stage such that the ICM at implantation consists of primitive endoderm (the outer layer) and primitive ectoderm (the inner cells) (Sherman,1977). By Day 6, the structure known as the egg cylinder has developed. During egg cylinder formation, a layer of primitive endoderm cells spreads over the inner surface of the trophectoderm forming the parietal endoderm layer. A second layer of primitive endoderm, known as visceral endoderm, surrounds the embryonic ectoderm of the egg cylinder. The embryonic ectoderm will eventually give rise to the three germ layers of the embryo (that is, embryonic ectoderm, endoderm and mesoderm) (Gardner and Rossant, 1979). There is also a proliferation of the polar trophectoderm cells

overlying the ICM, giving rise to the ectoplacental cone (epc) (Copp, 1979). This is an invasive tissue, invading the maternal deciduum. The resultant hemorrhage and pooling of blood around the epc cells probably establishes an early nourishment of the embryo, and the epc eventually forms part of the placenta. The mural trophectoderm cells begin the transformation to giant cells, via endoreduplication of their DNA (Copp,1978). Between the epc and the embryonic ectoderm lies the remaining tissue type found in the 6 day egg cylinder, the extraembryonic ectoderm (also surrounded by visceral endoderm). This tissue is also derived from the trophectoderm, and will give rise to the chorionic ectoderm, another placental component (Rossant and Lis, 1979a; Rossant, et al., 1983). This early post-implantation development is diagrammed in Figure 2.

Cell fate is determined by descriptive studies and cell lineage studies. Such studies have produced the plan of cell lineage relationships in early mouse embryogenesis diagrammed in Figure 3 (Rossant and Papaioannou, 1977). Cell lineage studies have made extensive use of experimental chimeras.

Chimeras in embryological studies

In Greek mythology, the chimera was the monster reputed to have the head of a lion, the body of a goat, and the tail of a serpent or dragon (Larousse,1966). In modern biological usage the term chimera is used to describe an animal which is a special type of genetic mosaic. Mosaics are animals in which two or more genetically distinct populations of cells co-exist. In a chimera, the two populations of cells have their origins in different animals. Chimeras do occur naturally in a variety of mammalian species, arising usually by placental vascular connections and cell mixing during twin pregnancy (Gardner,1968) but chimeras are most often a product of experimental manipulation.

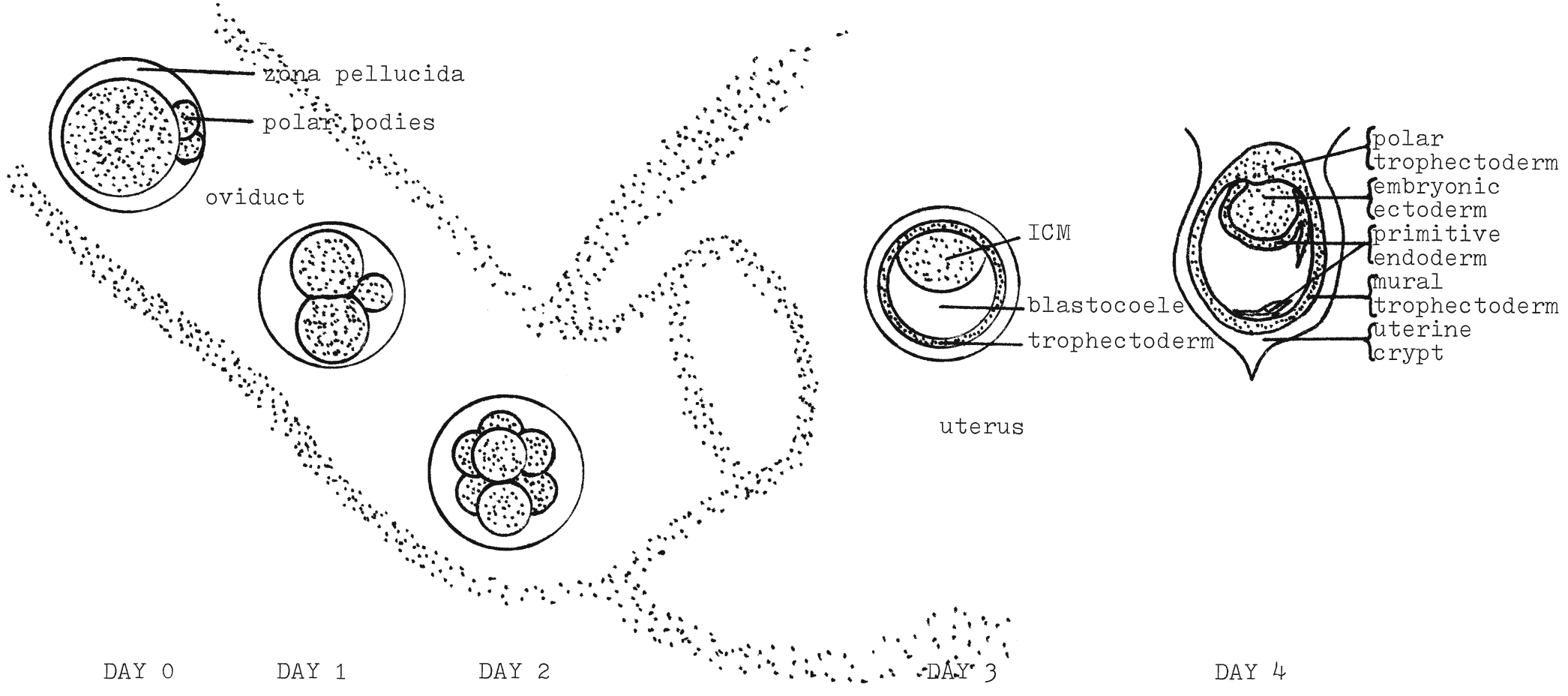


FIGURE 1 PRE-IMPLANTATION DEVELOPMENT OF MOUSE EMBRYOS

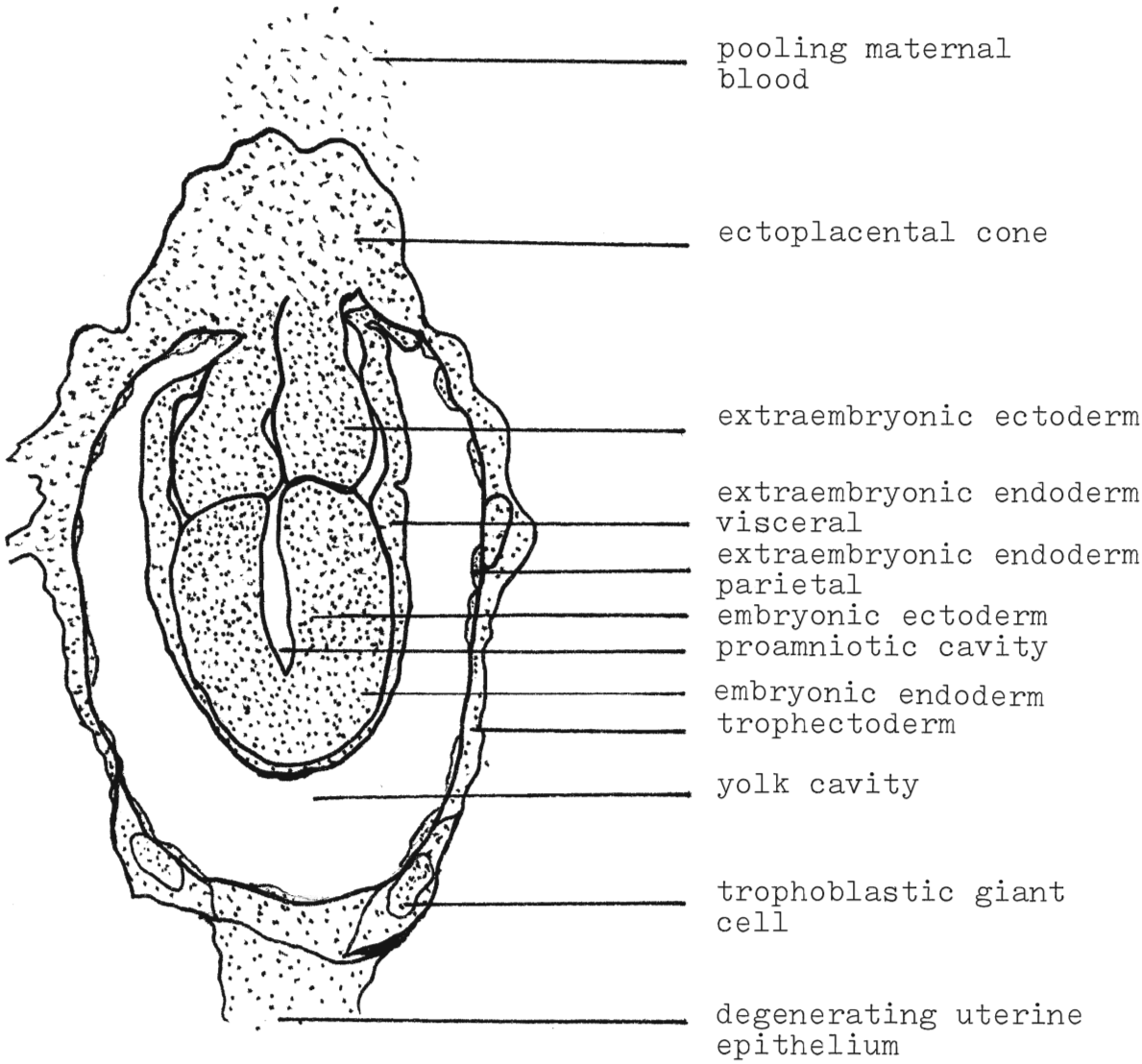


FIGURE 2 AN EGG CYLINDER, AROUND 6 DAYS DEVELOPMENT

redrawn from Snell and Stevens, 1966

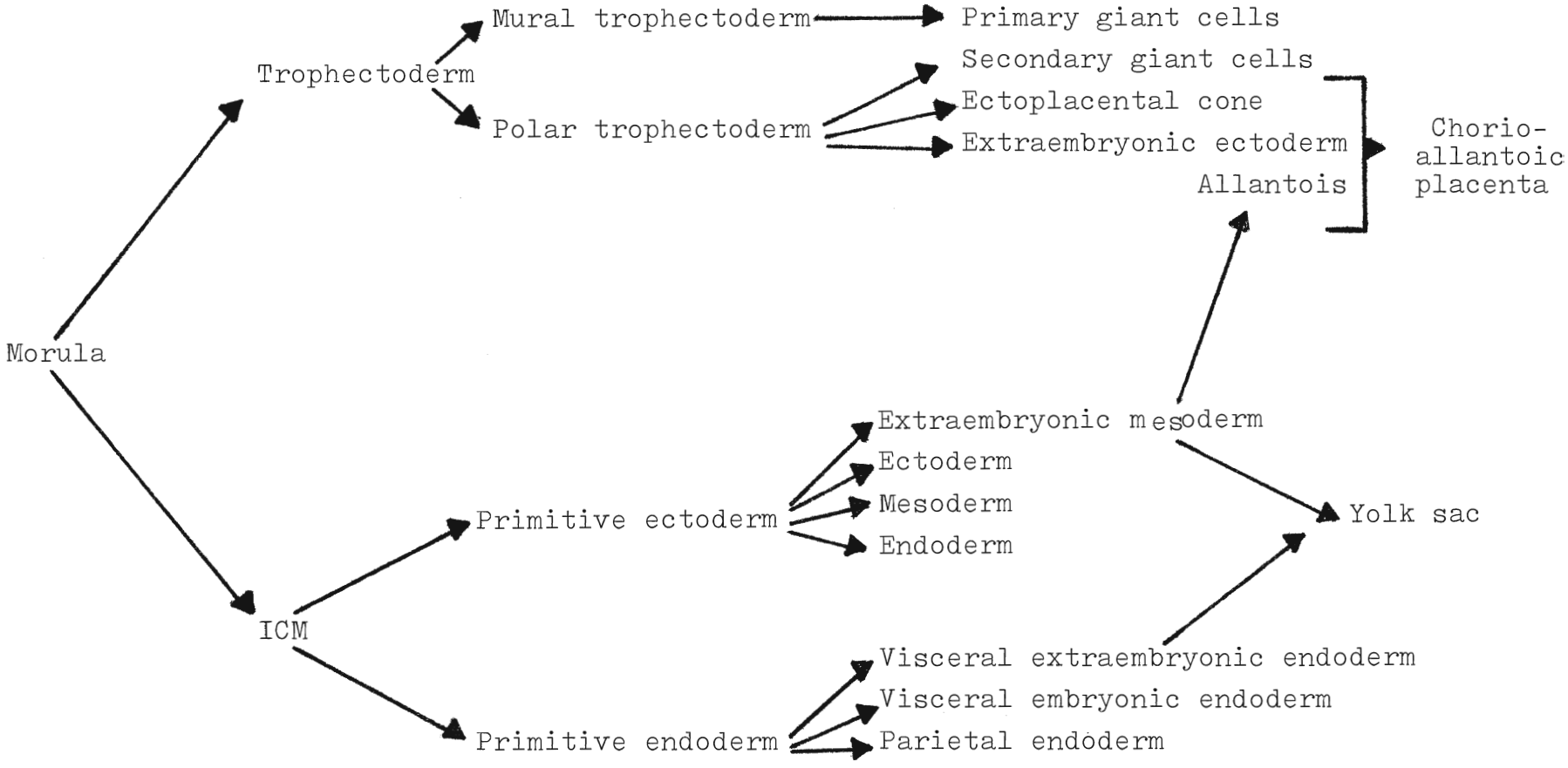


FIGURE 3 CELL LINEAGE RELATIONSHIPS IN EARLY MOUSE EMBRYOGENESIS

from Rossant and Papaioannou, 1977

In 1961, Tarkowski first reported the production of mouse chimeras following aggregation of 8-cell embryos and Mintz (1962, 1964) refined the technique for aggregating early morulae. In these studies the critera used for judging chimerism in the offspring was the production of intersexes and coat color mosaicism. Chapman et al. (1972) developed a valuable enzyme marker system such that chimeras began to be used as tools for investigations into various developmental questions. The enzyme glucose phosphate isomerase is a dimeric enzyme which is a very useful marker due to its widespread tissue distribution and the existence of electrophoretically variant forms (Carter and Parr, 1967). Homozygotes (Gpi-1b/Gpi-1b or Gpi-1a/Gpi-1a) produce either the faster migrating BB form of the enzyme or the slower migrating AA form. Heterozygotes (Gpi-1a/Gpi-1b) produce both AA and BB enzyme molecules in each cell as well as a hybrid form of the enzyme consisting of one A subunit and one B subunit. A chimera, made by aggregating a homozygous Gpi-1a/Gpi-1a embryo with a homozygous Gpi-1b/Gpi-1b embryo, will have two populations of cells, one producing AA enzyme molecules and the other producing BB molecules. AB enzyme molecules would not be found in any tissue samples from such a chimera unless cell fusion had occurred during development of that tissue. GPI is now widely used as the marker enzyme for detecting two cell populations in presumptive chimeras in many studies.

As Markert and Petters (1978) point out, thousands of healthy chimeric mice have now been produced. At the time of their study, the chimeras had all been derived from two embryos, and they then reported production of chimeras from aggregations of three 8-cell embryos. One triple chimera, with black, white, and yellow patches in its coat was produced from these aggregations (as well as offspring with mixtures of two coat colours, and single coat colours) indicating that at least three cells of the ICM had participated in development of the

embryo proper. They next went on to show that four 8-cell embryos could be aggregated with successful development of octaparental mice (Petters and Markert, 1980).

Development has been completely successful then, following aggregations of as many as four embryos with each other at the 4-cell stage, the 8-cell stage, or at the early morula stage. Although the aggregate morulae, and the blastocysts which develop from them are notably larger than normal embryos at these stages, the chimeric offspring are not giant mice, but are of normal size. Aggregate embryos are thus capable of size regulation. Lewis and Rossant (1982) demonstrated that this regulation occurred between 5.5 and 6.5 days development; that is, shortly after egg cylinder development. Aggregations between 2.5 day morulae and 3.5 or 4.5 day ICMs have also been successful and were the first proven cases of chimeras made through the association of cell types from two distinctly different stages of development (Rossant, 1975; Rossant and Lis, 1979a and b).

While the aggregation technique for producing chimeras has its place in developmental studies, a second, more sophisticated technique for producing chimeras early in embryogenesis has been developed. Gardner (1968) used a micromanipulator assembly to inject embryonic cells into the blastocoelic cavity of 3.5 day embryos. While this was a more difficult technique than aggregation, it allowed studies of a more precise nature, in which fewer cells were added to embryos or cells from a wide variety of developmental stages were mixed. Successful chimera production following blastocyst injection in experiments such as one (Gardner and Rossant, 1979) in which extraembryonic endoderm cells from 5 and 6 day embryos were injected into 3.5 day embryos indicate that the blastocyst can exert a regulatory effect over added cells also, even when a considerable degree of temporal asynchrony exists (Rossant, et al., 1978). An

even more remarkable example of regulation by the blastocyst will now be considered.

Cells from cell lines derived from malignant tumors have been injected into blastocysts. In some cases, regulation of the tumor derived cells occurred within the embryonic environment such that healthy, tumor free animals were born. Coat and eye pigmentation present in these animals readily indicated chimerism. That is, the originally malignant cells had been switched to normal pathways of differentiation through their association with the normal embryonic cells within the uterine environment. The tumors from which the cell lines were derived are known as teratocarcinomas, and the normalisation of these tumor derived cells is of prime interest in this study.

TERATOCARCINOMAS AND PROPERTIES OF EMBRYONAL CARCINOMA CELLS

Origins of teratocarcinomas

Spontaneous teratocarcinomas are tumors which have their origins in germ cells. These tumors were reported in the ovaries or testes of humans in the 19th century, and have long been intriguing as they characteristically contain an array of differentiated cell types. The tumors routinely contain tissues derived from all three embryonic germ layers. Illmensee and Stevens (1979), for example, display a photomicrograph of a sectioned tumor in which cartilage, bone with marrow, adipose tissue and muscle fibres, all of mesodermal origin, as well as neural tissue from ectoderm and glandular epithelium from endoderm have been identified. Additionally, in humans the tumors can contain such organized structures as hair and teeth. As gruesome as these tumors sound, if they contain only these differentiated cell types, they are benign and are known as teratomas. A teratocarcinoma is a malignant tumor however, with the potential for continued growth and metastasis in humans. (In the earlier literature, a teratomatous tumor is referred to as a teratoma, without distinguishing between those which were benign and those which were malignant.) The malignancy of these tumors is due to retention of an undifferentiated, proliferating population of stem cells, known as the embryonal carcinoma cells (reviewed by Illmensee and Stevens, 1979 and Martin, 1980). Because these cells have the potential to differentiate into a variety of cell types, they are referred to as multipotent or pluripotent cells. In this regard, they are similar to certain of the cells of the early mouse embryo.

It was largely the work of Leroy Stevens in the 1950s and 1960s which established the origins of teratocarcinomas. In 1953, Stevens noted testicular teratomas in mice of strain 129. The tumors arose early and could be found in

the fetuses by Day 15. They were localized exclusively in the seminiferous tubules of the testis, which contain the primordial germ cells. At this early stage, the fetal tumors in these tubules resembled 5 day embryos; that is, early egg cylinders. It seemed possible that the tumors might have arisen from the germ cell precursors which are set aside early in development (by Day 8) and which have migrated to the fetal genital ridges by Day 12. Indeed, when male genital ridges were grafted to the testes of histocompatible adult males a high incidence of grafts became teratomas. Finally, the germ cell origin of these tumors was firmly established by genetic manipulations, employing the mutant known as steel. Mice homozygous for steel completely lack germ cells. This mutation was introduced into the strain 129 subline with the high incidence of spontaneous tumors and recipients of homozygous steel genital ridges did not develop the tumors (reviewed in Illmensee and Stevens, 1979).

The marked similarity of the fetal tumors in the strain 129 seminiferous tubules to an early embryo suggested to Stevens that the tumors might also be inducible by grafting early embryos to ectopic (extrauterine) sites. He initially grafted 2-cell embryos to histocompatible adult testes and later (Stevens, 1969) grafted blastocysts and 6 day egg cylinders. Following grafting he observed normal embryonic development at the graft site for about one week. Then the grafts became disorganized and a small proportion eventually grew into typical teratomatous tumors. Those tumors retained an undifferentiated stem cell population and were readily transplantable from one host to another, such that they could be maintained long past the time when they would kill their original host. Stevens designated these induced, transplantable teratomatous tumors OTT.

From his work Stevens concluded that teratocarcinomas can arise either from germ cells in the fetal genital ridges, or from pluripotent cells in the early

embryo. As shown in Figure 4, embryos grafted at 5 through 7 days gestation (at the early egg cylinder stage especially) are particularly efficient at producing teratocarcinomas. By Day 8, a growth of differentiated tissues usually results which does not contain undifferentiated cells (Stevens, 1970). Then, at 11 days through 13 days gestation, grafts of male fetal genital ridges produce teratocarcinomas at a high frequency, but beyond 13 days, the incidence of the tumors falls off sharply. This is thought to be the result of a maturation of the primordial germ cells which prevents their continued tumor production (Illmensee and Stevens, 1979).

Just as Stevens had noted an increased incidence of testicular teratomas in male mice of strain 129, he eventually found a strain of mice with a high incidence of ovarian teratomas (Stevens and Varnum, 1974). Some of the eggs in the ovaries of these mice were behaving as if they had been fertilized and were undergoing the initial steps of normal embryogenesis. Most of these apparently died before primary germ layer formation. However, some progressed in an organized fashion through the blastocyst stage to the early egg cylinder stage, with recognizable endoderm and ectoderm layers. At this point the growths became disorganized. The tumors seen were classical teratomas with cartilage, bone, adipose tissue, hair, and so on.

In summary, Stevens and Varnum (1974) point out that 4 sources of teratomatous tumors were now known in mice. These are:

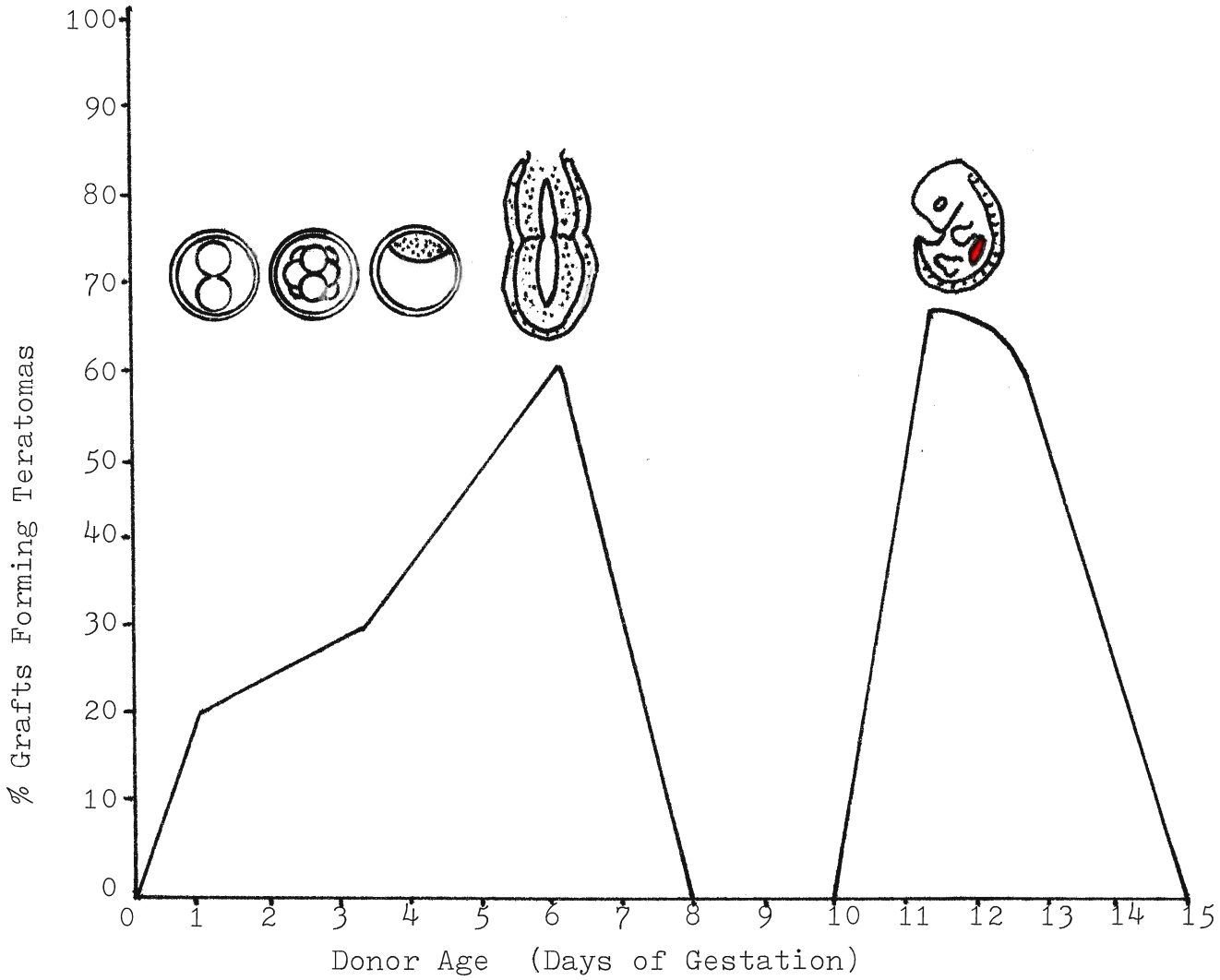
1. spontaneous testicular tumors, arising from the primordial germ cells in the seminiferous tubules

2. spontaneous ovarian tumors, arising from parthenogenetic activation of oocytes within mature ovaries

3. experimentally induced tumors, arising from grafting 11 to 13 day male fetal genital ridges, containing germ cells, to various sites, especially the testis

4. experimentally induced tumors, arising from grafting male or female early egg cylinders, containing pluripotent embryonic cells, to ectopic sites.

FIGURE 4



Experimental teratomas are induced by grafting an early embryo or the genital ridge of a fetus to an extrauterine site in an adult mouse. A 6 day embryo or an 11 to 13 day genital ridge is most likely to produce a teratoma. (Illmensee and Stevens, 1979)

Properties of embryonal carcinoma cells

The embryonal carcinoma cells are the undifferentiated, proliferating stem cells which confer malignancy on the teratomatous tumors in which they persist. These stem cells make serial transplantation of the tumors possible, and a number of cultured EC cell lines have now been established from various transplantable tumors.

The embryonic counterpart of EC cells

Martin (1980) has given some consideration to the normal embryonic equivalent of EC cells. The ICM of the pre-implantation embryo consists of pluripotent cells. At the time of the first differentiation of the ICM cells, two cell types arise: the pluripotent primary ectoderm which will give rise to the fetus, and extraembryonic endoderm of limited potential (Gardner and Rossant, 1979). In vitro, when EC cells form embryoid bodies, they give rise to an outer layer of endodermal cells which surround the EC core. This observation suggests that EC cells might most closely resemble the cells of the ICM of the early embryo (Martin, 1975; Martin and Evans, 1975; Martin, et al., 1977). However, a report by Diwan and Stevens (cited by Martin, et al., 1978) that isolated embryonic ectoderm grafted to an extrauterine site will itself give rise to a teratocarcinoma suggested that EC cells might actually correspond most closely to the primary ectoderm. Accordingly, Martin, et al.(1978) compared the protein synthesis patterns in morulae to that of blastocysts, or their ICMs, to the pattern seen in EC cells. Differences in the electrophoretic profiles of [^{35}S]-met labelled proteins were seen between 8-cell and later stage embryos. The EC profile differed markedly from the pattern seen in cleavage stage embryos and showed greatest similarity to the isolated ICM profile, although it contained a

band missing from the ICM profile. The profiles from later ICMs closely matched the EC cell profile, including the previously missing band. It was the profile from the isolated embryonic ectoderm from these ICMs which contained the band characteristic to several EC cell lines, and therefore Martin and her colleagues have concluded that EC cells might be most closely analogous to the pluripotent embryonic ectoderm cells of the early post-implantation ICM. This is probably now the most widely accepted view.

In vitro behavior of EC cells: The induction of differentiation by retinoic acid

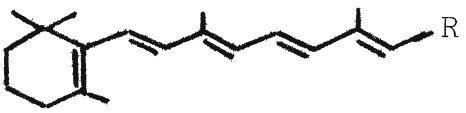
EC cells may be valuable substitutes for embryonic cells in in vitro investigations of a variety of developmental events. Mention of the ability of EC cells to differentiate spontaneously in vitro has already been made. In suspension culture, aggregates differentiate into the embryoid bodies investigated by Martin, amongst others. Additionally, differentiation of EC cells can be induced in vitro by various chemical inducers, particularly retinoic acid.

Retinoic acid is a vitamin A derivative. Its structure is shown in Figure 5. Vitamin A is known to have many biological effects, defined largely by nutritional deprivation studies, which include effects on growth and fertility, and on the normal development of skin, epithelia, teeth and bones. While essential to good health, vitamin A in excessive amounts is known to have toxic properties, and is a potent teratogenic agent (Hogan, 1979).

Aware of the effects of retinoic acid on epithelial tissues, and on various tissue remodelling processes (Wilson and Reich, 1978; Strickland and Mahdavi, 1978) Strickland and Mahdavi asked if retinoids might promote the differentiation of EC cells in vitro. They tested the effect of retinoic acid on F9 cells, an EC cell line which undergoes very limited differentiation normally. In response to 10^{-7}M RA, morphological changes were seen in the F9

FIGURE 5

Basic structural formula of retinoids



R= $-CH_2OH$: parent substance: trans-vitamin A alcohol

R= -CHO: trans-retinal (vitamin A aldehyde)

R= -COOH: trans-retinoic acid (vitamin A acid)

cultures within 24 hours, and eventually the cultures contained large flat cells closely resembling the presumptive endoderm derived from embryoid bodies.

P19 is another EC cell line which does not readily differentiate in vitro. The only differentiated cells to appear spontaneously resemble extraembryonic endoderm cells, and stem cells always persist even when these cells appear. More extensive differentiation of P19 cells was initially induced by dimethylsulfoxide (DMSO). Differentiation of cells in response to DMSO was first noted in 1971 by Friend and her coworkers when cells of a tissue cultured line of mouse virus-induced erythroleukemic cells in medium containing 2% DMSO differentiated and began secreting hemoglobin. McBurney found that P19 cells also differentiate in response to DMSO (McBurney, et al., 1982). When P19 aggregates were exposed to DMSO in suspension cultures for 4 to 5 days and then plated out, differentiation became apparent within 2 days. The cultures contained contracting muscle cells and fibroblast-like cells. The muscle cells in DMSO treated cultures contained muscle specific myosin, identified in an immunofluorescence assay (Edwards, et al., 1983).

If the P19 aggregates were exposed instead to retinoic acid for 5 days they differentiated into neurones and glial cells. Jones-Villeneuve, et al. (1982) determined that the differentiation of P19 cells into neuronal cells was dependent on the retinoic acid concentration. At concentrations greater than $5x10^{-8}$ M for at least 24 hours before plating all cultures contained cells with neuron-like processes by 72 hours after plating. Immunofluorescence assays have confirmed the presence of neurofilament protein, and elevated levels of choline acetyltransferase and acetylcholinesterase have also been found in the RA treated cultures containing presumptive neuronal cells (Jones-Villeneuve et al., 1983).

McBurney's emphasis has been directed towards determining RA treatment

conditions which promote differentiation of P19 cells along specific routes. In assessing the response of cultures to various concentrations of the drug for various times, he has recorded the appearance of neuron-like cells, for example, in aggregate outgrowths. However, confirmation of persistence of undifferentiated EC cells, despite differentiation of other cell types may be of considerable importance in other types of experiments.

Solter and Knowles produced a monoclonal antibody in response to immunization of a mouse with EC cells which reacted with an antigen present on undifferentiated EC cells, but not on their differentiated derivatives (Solter and Knowles, 1978; Solter, et al., 1978). Embryos from various stages were tested for reactivity, and antibody was not bound to unfertilized eggs, single, 2 or 4-cell embryos. Eight-cell embryos began to express the antigen, while morulae and ICMs from blastocysts bound the antibody quite efficiently. This monoclonal antibody is specific, therefore, for an antigenic determinant present on EC stem cells and on embryonic cells from specific stages. Solter designated this antigen stage-specific embryonic antigen 1 (SSEA-1) and potentially it could serve as a marker for undifferentiated EC cells persisting in RA treated cultures.

McBurney has recently employed a similar antibody to monitor loss of stem cells from his cultures. The monoclonal antibody AECA1-9 appears to detect an antigen closely related to the antigen detected by the antibody to SSEA-1 (Harris, et al., submitted for publication). An indirect immunofluorescence assay demonstrated that 2 days after plating treated aggregates a very high level of antigen expression is evident (Jones-Villeneuve, et al., 1983) indicating initial persistence of undifferentiated stem cells in retinoic acid treated cultures.

As McBurney points out, (McBurney, et al., 1983) DMSO is of no

physiological significance. It may exert its influence through various rather non-specific solubilizing effects. But retinoids are naturally occurring. In a study into possible mechanisms of action of RA, Jetten and Jetten (1979) were able to demonstrate the presence of a cytoplasmic binding protein for RA in various EC cell lines. The existence of binding protein is shown through binding of [^{3}H]-retinoic acid to a protein component by a cytosolic supernatant from the EC cells. The supernatant from the nuclear fraction did not bind [^{3}H]-RA, but in cells which had been preincubated with [^{3}H]-RA the nuclear fraction was found to contain [^{3}H]-RA. Thus, it was postulated that RA must first be bound to a cytoplasmic binding protein and that this complex is transported to the nucleus, a model reminiscent of the proposed mechanism for transport of steroid hormone-receptor complexes to the nucleus. These complexes are then thought to interact in some way with chromatin.

Schindler, Matthei and Sherman (1981) isolated two mutant clones which did not respond to RA, and their data suggest that the failure to differentiate was due to a lack of binding of the RA to a cytoplasmic RA binding protein (cRABP). Therefore, this complexing of RA to cRABP is likely an essential step in the sequence leading to RA induced differentiation of EC cells.

How might retinoic acid bring about differentiation of different cell types in a developing embryo? McBurney has suggested that an RA concentration gradient might play a role. In the initial experiments in which P19 cells were treated with retinoic acid, cardiac muscle was rarely observed and skeletal myoblasts were never seen. Rather, these cultures contained cells of the neuronal lineage. But when much lower doses of RA were used, they were able to show that the differentiated cell types formed were dependent on the RA concentration used. At 10^{-9}M RA, an abundance of cardiac muscle was seen, while at 10^{-8}M skeletal muscle predominated, and neurones and astroglia appeared at

10^{-7} M RA. In contrast to the first low dose studies these cells were cultured for many days after treatment with the low RA doses to see the effects, and large numbers of undifferentiated cells persisted for some time in these low dose cultures (Edwards and McBurney, 1983). McBurney has suggested an hypothesis based on these results which proposes that "gradients of retinoids may play some role in the developmental decisions taken by these embryonic cells" (McBurney, et al., 1983).

Participation by EC cells in normal differentiation in chimeras

EC cells, originating from the pluripotent cells of the embryonic ectoderm of the early egg cylinder, appear to retain many similarities with these cells of the early embryo, as judged by their ability to undergo differentiation to a wide variety of cell types in vitro. However, the ultimate test of normality of EC cells would be the demonstration of the ability of these cells to participate in normal development in a chimera.

In 1974, Brinster reported the recovery of one chimera (judged by coat pigmentation only) following injection of cells from embryoid bodies into blastocysts (Brinster, 1975). This was the first demonstration that the malignancy of the stem cells could be reversed. In 1975, Mintz and Illmensee reported a more detailed study into the properties of the cells within embryoid bodies of OTT 6050. Core cells were injected into blastocysts and they reported recovery of chimeras, at a low frequency, which were tumor free and demonstrated chimerism throughout a wide variety of tissues.

Meanwhile, attempts were underway to establish in vitro tissue cultured cell lines from the teratocarcinomas maintained as in vivo transplantable tumors. For example, Lehman, et al. (1974) used cell suspensions from embryoid bodies from the same OTT 6050 tumor to initiate tissue cultures. The primary cultures consisted of two cell types: flat epithelial cells and dense colonies of cells. Within a few days additional cell types could be seen while the dense colonies persisted. These dense colonies contained cells of identical morphology to the cells of the embryoid body cores, and were assumed to be the EC cells. These cells, after cloning and subcutaneous injection into mice, gave rise to typical teratocarcinomas while the differentiated cells did not.

One possible application of cultured EC cells was in establishing animal models for human disease. The Lesch-Nyhan syndrome in humans is characterized

by severe neurological symptoms as well as other problems. It is the result of a deficiency of the X-linked gene for hypoxanthine phosphoribosyl transferase (HPRT). While the HPRT gene is also X-linked in the mouse, HPRT- mice have not been found, and in fact no animal model exists for Lesch-Nyhan syndrome. Dewey, et al. (1977) attempted to introduce the defective gene into mice using HPRT- EC cells as the vehicle. While chimeras were produced in which HPRT- cells made up a sizeable fraction of certain tissues (which showed lower than non-chimeric control enzyme levels) no animal showed any disease symptoms. The EC cells used were XO and trisomic for chromosome 6, and were unlikely to colonize the germ line. This occurrence of karyotypic abnormalities in cultured EC cell lines is a recurring problem.

Papaioannou and her co-workers also tested the developmental potential of various cultured EC cells in blastocyst injection experiments (Papaioannou, et al., 1975, 1978). They found that the rate of chimera production was very low, and tumors were found in most of the chimeric offspring while the contribution by the EC cells to normal tissues was sporadic and very limited, usually restricted to trace amounts only. Karyotypic abnormalities were detected in all the cell lines used in this study, and these abnormalities may have affected the capacity of the cells to participate in normal embryogenesis.

Both these groups saw the establishment of EC cell lines with normal karyotypes as an important next step in assessing the ability of these cells to participate in normal development (Cronmiller and Mintz, 1978). The validity of using cultured EC cells as in vitro models for various aspects of embryogenesis, as well as their potential use as carriers of selected mutations into mouse strains was rather dependent on a demonstration of a complete recovery of normal function in vivo (Papaioannou, et al., 1978).

Performance of diploid EC cell lines

The METT-1 EC cell line

In 1981, Mintz reported success. She had derived an EC cell line which retained a normal female karyotype. Three to five cells only were injected into blastocysts in a study of this EC cell line's ability to differentiate normally in the embryonic environment (Stewart and Mintz,1981). Only 13% of the offspring from these experiments were obvious chimeras by coat colour. The mouse showing the highest EC contribution to the coat (judged at 85%) was used for extensive GPI analysis. All tissues tested showed extensive contribution by the EC cells, ranging from an estimated 10% in the pancreas to 65% in the brain, and averaging 43%. Nine chimeric females were selected for a test breeding program. The appearance of an agouti coat colour in any of their offspring would indicate that an EC cell had differentiated into a functional oocyte. Out of 329 offspring, three agouti babies were found, all offspring of one chimeric mother, with 65% agouti in her coat. Two of these were females, who subsequently also produced agouti offspring in the F2 when mated with the male agouti from the F1. In this study, only one tumor bearing animal was found. It was assumed that one of the cells injected into the blastocyst had escaped regulation and had continued malignant proliferation. Because completely normal embryogenesis, including colonization of the germ line in some cases, was apparently the route taken by a large number of the injected cells, the cell line was designated METT-1, for "Mouse Euploid Totipotent Teratocarcinoma" cells. The "1" was appended optimistically, signifying the first of perhaps a number of such EC cell lines that might perform equally well once more euploid lines were available.

The P10 EC cell line:

The P10 EC cell line was derived by McBurney and Strutt (1980). Tumors were induced by grafting 6.5 day C3H strain embryos to the testes of C3H hosts and one EC cell line isolated was found to have a normal female karyotype. This line was designated P10.

The in vivo developmental potential of the P10 line following blastocyst injection has been assessed by Rossant (Rossant and McBurney, 1983; and Rossant, unpublished observations). In these experiments, 57% of live born offspring were obvious chimeras by coat colour (plate 1). These animals were all healthy and normal, with no sign of tumor development. GPI analysis of several chimeras indicated widespread P10 contribution throughout the tissues of these mice although the highest average overall tissue contribution was only 42%. Eleven female chimeras were selected for a breeding program, but no colonization of the germ line was noted in over 300 offspring. This represents a fairly restricted breeding program. Recall that Mintz found only three METT-1 derived progeny among about the same number of offspring, and they were all progeny of one chimeric mother. Therefore, these negative results do not necessarily exclude a P10 contribution to the germ line.

The recovery rate of live born offspring was rather low in these experiments, at 58%. This suggested that some P10 chimeras were not surviving to term. Accordingly, several pregnancies were examined pre-term, and abnormal fetuses were found. The abnormalities frequently included an open neural tube, and an unfused allantois was noted in one fetus as well as complete disorganisation in another. The average P10 contribution to these embryos was much higher that that seen in the live born offspring and the highest P10 contributions were almost invariably associated with the most obvious developmental abnormalities. When the outcome of full term pregnancies alone is

Plate 1 P10 Blastocyst Injection Chimeras

Chimeric mice, produced by injecting 15 to 20 P10 cells into the blastocoelic cavity of a Day 3 embryo. The pigmentation in the eyes and coat is a visual indicator of P10 contribution. GPI marker enzyme analysis indicated that live born chimeras such as these demonstrated widespread colonization of tissues by the P10 cells, but overall P10 contribution did not exceed 50%. While these mice remained healthy and tumor free, abnormal embryos, with a higher proportion of P10 cells, were observed in pregnancies examined pre-term.

Photo courtesy J. Rossant

considered, P10 appears to be a candidate to join METT-1 as another example of a karyotypically normal EC cell line which can participate extensively in normal embryogenesis following blastocyst injection (Rossant and McBurney, 1983). P10 has been the most successful EC cell line tested so far in its ability to produce normal live born chimeras (Papaioannou and Rossant, 1983). However, upon consideration of the loss of abnormal fetuses during gestation, it seems reasonable to suspect that high levels of P10 contribution might be incompatible with normal development. That is, when the P10 cells constituted a majority population, their proliferation and differentiation may not have been completely regulated. In order for normal development of P10-embryo chimeras to proceed, a majority population of normal embryonic cells may be required to provide the proper developmental framework.

This P10 study differs from the study assessing the _in vivo_ developmental potential of METT-1 in that pre-term pregnancies were examined. The development of METT-1 embryo chimeras was not assessed during gestation and the rate of live births seems rather low, at 31%. It is possible that abnormal METT-1 chimeras might have been lost during development as well. The recovery of abnormal P10 chimeric fetuses indicates that being euploid is not a guarantee of successful developmental performance, and suggests that even euploid EC cells are not the complete equivalent of normal embryonic cells. This conclusion is given further support when the experiments involving injections of P19, McBurney's other euploid EC cell line, are considered.

The P19 EC cell line

The P19 EC cell line was derived by McBurney and Rogers (1982). Again, tumors were induced by grafting C3H egg cylinder stage embryos to the testes of host animals and the P19 cell line was established directly from the primary

tumor. McBurney attempts to isolate cell lines as rapidly as possible from newly induced teratocarcinomas and this may be the reason for his success in establishing EC cell lines which have not undergone karyotypic modification.

The in vivo developmental potential of a clonal subline of P19 (P19S18) has been assessed by Rossant (Rossant and McBurney, 1982). When 15 to 20 P19S18 cells were injected into blastocysts the rate of chimera production was high but abnormalities were evident in 9.5 day embryos. The chimeras assessed as developing normally were almost always those with lower P19 contributions than that seen in the abnormal chimeras.

At 12.5 days gestation, a large number of resorption sites were found, indicating high embryo loss. The live chimeric embryos contained relatively low P19 contributions, usually less than 50% in the tissues analysed. Only four live born chimeras were recovered, and all had obvious tumors on the head along with low level P19 contributions to at least some normal tissues. Therefore, incomplete regulation of presumably homogeneous cloned P19S18 cells had occurred.

Following publication of her work, Papaioannou (Papaioannou, et al., 1978) was criticized for the large numbers of EC cells injected. From 20 to 40 EC cells were injected into blastocysts in those experiments, probably in an attempt to facilitate recovery of chimeras with significant EC contributions. Illmensee, Martin, Mintz, and Pierce have all expressed the view that the tumors present in the chimeric mice recovered in these experiments probably reflected incomplete regulation of such a large number of EC cells imposed upon the blastocyst. If fewer cells had been injected, these workers all predicted complete regulation (Martin, 1980; Pierce, et al.,1979; Illmensee and Stevens, 1979; Dewey, et al., 1977). Because P19 cells produced chimeras at such a high rate, it seemed feasible to test the outcome of injecting a single EC cell into

a blastocyst. When a single P19S18 cell was injected, an improvement in live embryo recovery beyond midgestation was noted but only 15% were chimeric and most showed the same types of abnormalities in the head region that had been seen previously. Only one live born chimera was recovered. This animal had a large tumor on the head. So, although P19 is a karyotypically normal EC cell line, it appears to be incapable of completely normal embryogenesis (Rossant and McBurney, 1982).

Summary: Comparison of the developmental potential of euploid EC cell lines tested to date following blastocyst injection

A total of six euploid EC cell lines have been tested for normal developmental potential following injection into blastocysts. We have considered three so far: METT-1, P10 and P19. Additionally, Papaioannou and her co-workers (1979) derived and tested three others. Only one (C145b) formed any chimeras, at a rate less than 1%, and in these the EC contribution was only to the yolk sac tissues. Therefore, these euploid EC cell lines, which among the various EC cell lines might be expected to most closely resemble normal embryonic cells by virtue of their normal karyotype, behave quite differently from each other, and also quite differently from ICM cells following blastocyst injection. In injections of normal ICM cells into blastocysts, 90 to 100 % of the offspring are healthy chimeras. Even when a single ICM cell is injected into a blastocyst at least 25% of the offspring are chimeric (Papaioannou and Rossant, 1983). Some EC cells do not colonize the embryos at all, while P10 and METT-1 have produced normal live born chimeras at a fairly high rate, though not at the rate seen following injection of an ICM. Abnormalities of fetal development during gestation have been observed following P10 injections, and one METT-1 chimera had a tumor. Abnormal development and tumors have never been

seen in chimeras developing from blastocysts injected with ICMs. P19 also gave rise to chimeras at a very high rate following blastocyst injection but the great majority were abnormal and did not survive. Live born chimeras all had tumors (Papaioannou and Rossant, 1983). Clearly then, these EC cell lines differ in their response to the blastocyst environment. Rossant and McBurney (1983) have considered possible explanations for these differences in response. They compare P10 and P19 and point out firstly that the normality of the chimeras produced by the two lines is apparently not a simple function of the degree of EC contribution. Within the chimeras produced by both lines, there may be a correlation between the degree of EC contribution and the degree of abnormality. However, P10 chimeras with EC contributions as large as those seen in abnormal P19 chimeras were apparently developing normally. It has been noted that P10 cells differentiate quite readily in vitro, whereas P19 cells are less likely to do so, and stem cells always persist in the culture. In producing any in vitro EC cell line, cells with the property of continued proliferation are selected. Stem cells in the early embryo do not proliferate indefinitely; rather, they differentiate. P10 cells may not have diverged in culture as far from their normal counterparts as have P19 cells, and may have retained a greater ability to respond to normal developmental signals within the blastocyst environment (Rossant and McBurney, 1983). The observations on EC-embryo chimeras presented to this point suggest strongly that EC cells are not completely equivalent to normal pluripotent embryonic cells.

EC-embryo chimeras produced by the aggregation technique

The potential of the aggregation technique for making EC-embryo chimeras will now be considered. In 1980, two reports of successful aggregations between cultured EC cells and morulae were published. Stewart (1980) and Fujii and Martin (1980) both found that while EC cells will aggregate successfully with cleavage stage embryos, with some variation in adhesiveness between the cell lines used, differentiated cell types will not form successful aggregates with morulae. The EC-morula aggregates developed into morphologically normal blastocysts in vitro.

Having demonstrated the feasibility of aggregating EC cells with 8-cell embryos, both groups went on to test the developmental potential of these aggregation chimeras in vivo. Stewart (1982) tested two cell lines, PSA/NG2 and PSA/TG12. Both lines are XO and trisomic for chromosome 6. Three to five EC cells were aggregated with two 8-cell embryos and very few of the embryos recovered during gestation or the live born animals were chimeric. In chimeras, EC contribution was found in the embryo proper and in the yolk sac mesoderm, and only occasionally to the yolk sac endoderm. The extent of this contribution is not given. No mention of abnormalities amongst the chimeric fetuses is made.

When NG2 cells were aggregated with only a single 8-cell embryo a striking difference in the outcome was noted in that most chimeric fetuses were "severely malformed" at 12 to 13 days. Further details are not given.

In his discussion, Stewart points out that aggregation is a simple alternative to the blastocyst injection technique for producing viable EC-embryo chimeras. The rate of chimera recovery was quite low however. He comments on the preferential colonization of the yolk sac mesoderm and suggests that the EC cells did not respond to the signals to differentiate endoderm. He does not comment on the recovery of abnormal fetuses, but his data suggest that when the

number of normal embryonic cells in the aggregates was decreased, the EC cells colonized the embryos more readily. Unfortunately, he does not give any indication of relative contributions, so we cannot determine if the abnormal embryos had higher EC contributions than the normal chimeras. It must be kept in mind that the EC cells were aneuploid, with a known lethal trisomy, when assessing the significance of the abnormalities. The presence of this trisomy in some of the cells of the developing chimeras may have adversely affected their development.

Fujii and Martin published a more detailed assessment of the developmental potential of EC-embryo aggregation chimeras in 1983. They anticipated that the aggregation technique might increase EC contributions in the chimeras because the EC cells are brought into association with the normal embryonic cells 24 hours earlier than in blastocyst injection.

They aggregated about 10 to 20 PSA-1 or PSA/NG2 cells with a single 8-cell embryo. At 10.5 days gestation, they observed a large number of abnormal fetuses, reinforcing Stewart's observations of abnormal NG2-embryo chimeras. The NG2 cells had colonized the embryos at a high rate: 79% were chimeric, but only one of these chimeras appeared to be developing normally. The abnormal embryos were classed as retarded and primitive structures only barely recognizable as fetuses. These abnormal embryos were heavily chimeric, usually with an EC contribution greater than 50%.

The effect of decreasing the EC component of the original aggregate was tested by aggregating the EC cells with two 8-cell embryos. The frequency of chimerism was decreased somewhat to 62% and abnormal chimeras were not nearly as abnormal as the previous set. No further improvement was noted when three embryos were included in the aggregates. These more normal chimeras had lower EC contributions than the previous set, generally less than 50%.

The rate of live born chimera recovery was low and the EC contribution in these animals was sporadic and limited, and one had a tumor. Thus, it appeared that aggregation was a very useful technique for producing chimeras at a high rate, with high EC contributions in each embryo, but most of these chimeras were being lost during gestation due to developmental abnormalities.

It seemed likely that the high EC contributions were incompatible with normal development. However, both PSA-1 and PSA1/NG2 are XO and trisomic for chromosome 6. The effect of the trisomy on development was of particular concern, and further aggregations were performed therefore, between nearly euploid EC cells and an 8-cell embryo. When LT1-2D cells (with an elongation of 1 chromosome #8 as the only known karyotypic abnormality) were aggregated with an 8-cell embryo, abnormal chimeras were again recovered. Fujii and Martin claim that their more normal karyotype has not given them an advantage, but their data chart suggests to me that the abnormal LT1-2D chimeras did not exhibit as severe a degree of abnormality as PSA-1 abnormal fetuses, despite high EC contributions in some cases.

Fujii and Martin conclude that aggregation is a simple and efficient alternative to blastocyst injection for making chimeras. They point out that in order to fully explore the developmental potential of an EC cell line, chimeras in which the EC cells predominate must be constructed. Their aggregation chimeras show high EC contributions, and their data suggest that high EC contribution correlates with abnormal development. It would seem then that these results support Rossant's hypothesis that normal embryonic cells must predominate in a chimera to program normal development, and therefore that EC cells do not have the same developmental potential as normal embryonic cells. However, certain qualifiers must be attached to this statement. Firstly, Fujii and Martin considered the possibility that mixing cells of different

developmental stages in an aggregate might adversely affect development. We have already seen evidence that EC cells are different from the cells of the 8-cell embryo. For example, as Martin determined, their protein synthesis pattern is quite different from that seen consistently in various cleavage stages, and correlates instead with the pattern seen in ICMs from later blastocysts. EC cells probably correspond most closely to the embryonic ectoderm cells of such ICMs. Fujii and Martin have some evidence for an adverse effect on development following aggregation of an ICM or embryonic ectoderm cells with an 8-cell embryo. (This conclusion was not completely clear cut from their data, however.) While the abnormalities seen in the EC aggregation chimeras were of greater magnitude and probably cannot be attributed solely to the mismatching of embryonic cell types in the aggregate, the role of this mismatching in promoting abnormal development is not entirely clear.

Secondly, chromosomal aberrations were present in all three cell lines tested in the study and may have contributed to the developmental difficulties experienced by the chimeras. The presence of a trisomy in two of the lines is probably the most serious deviation from the normal karyotype, and the other line was nearly euploid. Abnormalities were seen in all three types of chimeras, but the most normal EC cell line may have shown an improved developmental performance.

Thirdly, and perhaps most importantly, it is not clear from these aggregation studies if the high rate of abnormality is a consequence of the aggregation technique per se, or if abnormal, highly chimeric embryos can also be recovered following injection of these EC cells into blastocysts. A detailed study, including examination of preterm pregnancies, of the developmental potential of these EC cell lines following blastocyst injection has not been undertaken.

Questions remain then, concerning the potential of EC cells and ICM cells to participate in normal development following aggregation with 8-cell embryos.

At this point, a summary of certain properties of selected EC cell lines pertinent to this study might be useful. Table 1 illustrates the following relevant points:

1. Euploid EC cell lines vary in their ability to colonize the embryo following blastocyst injection. One EC cell line did not colonize the embryo at all and P10 was the most successful in terms of % live born animals which were chimeric.

2. Survival to term is low following injection of EC cells compared to injection of ICM cells. Again, P10 was the most successful line in terms of survival, and none of these P10 chimeras had tumors.

3. During midgestation, many abnormal EC-embryo chimeras were found. P10 produced the lowest rate of abnormal fetuses.

4. Aggregation produces high rates of chimeric offspring but also may produce high rates of abnormal offspring, perhaps due to the high EC contributions achieved. However, fully euploid EC cells have not yet been aggregated with 8-cell embryos, and available data on aggregation of ICMs with 8-cell embryos are insufficient to determine whether developmental difficulties might be partly due to mismatching of asynchronous cell types in the aggregates. Furthermore, it is very clear from the table that the only EC cell lines which have been tested for their developmental potential following aggregation have not been

fully tested for their developmental potential in a chimera following blastocyst injection. The limited injection data available for PSA-1/NG2 suggests that the rate of live born chimera production is very low following injection also, and that highly chimeric, abnormal embryos might also be produced and lost during gestation following injection.

TABLE 1

The in vivo developmental performance of selected EC cell lines in EC-embryo chimeras

Cell line or type	Karyotype	Method used to make chimeras	DEVELOPMENTAL PERFORMANCE				
			Midgestation % chimeric	Midgestation % chimeras abnormal	Survival to term	Term % survivors chimeric	Tumors
ICMs	Euploid	Bst. injection	N.A. (most survive to term)	N.A.	80%	90 to 100%	No
C145b	Euploid	Bst. injection	<1% (y.s.) only			0%	
METT-1	Euploid	Bst. injection	N.D.	N.D.	31%	13%	Yes (1)
P10	Euploid	Bst. injection	100%	40%	58%	57%	No
P19S18	Euploid	Bst. injection	69%	55%	36%	27%	Yes
PSA-1/NG2	XO,ts 6	Bst. injection Aggregation	N.D. 79%	N.D. 93%	? 13%	23% 29%	No Yes
PSA-1	XO, ts 6	Aggregation	58%	58%	N.D.	N.D.	N.D.
LT1-2D	1 #8 elongated	Aggregation	40%	83%	N.D.	N.D.	N.D.

N.A.: not applicable N.D.: not determined ts: trisomy y.s.: yolk sac

THE QUESTIONS RAISED IN THIS STUDY

The phenomenon of regulation and participation in normal development by cultured EC cells upon their return to the embryonic envirnonment is of particular interest in this study. The P10 and P19 cell lines seemed to be ideal candidates as EC cell lines which could be used to explore more completely the effects of aggregating EC cells with morulae. They are euploid and their response to the embryonic environment following blastocyst injection has been fully explored. It was hoped that by aggregating these cells with early morulae chimeras might be generated at an even higher frequency and that the EC cells would predominate in at least some of the chimeras such that the *in vivo* developmental potential of euploid EC cells could be more completely assessed.

The following questions are considered in this study.

1. Aggregation is an efficient technique for producing EC-embryo chimeras, but apparently produces a high level of abnormality. Is there really a difference in the rate of abnormal development produced by aggregation compared to blastocyst injection?

2. If so, does a high rate of abnormality correlate with higher EC contributions achieved in aggregation chimeras?

3. Alternatively, are there developmental problems associated with mismatching of cells from different developmental stages? If so, ICM-morula aggregates would also be expected to show abnormalities of development.

4. Can an improvement in the developmental performance of an EC cell line be induced by a pretreatment of EC cells with retinoic acid? If so, P19 cells, which seem to persist to some degree as stem cells in all chimeras, might be committed to differentiation and more normal development as a result of retinoic acid pretreatment.

MATERIALS AND METHODS

Mice and staging of embryos

Embryos were obtained from originally random bred CD-1 mice (Charles River, Quebec). For most experiments, it was necessary that embryos were homozygous for one or the other GPI (glucose phosphate isomerase) type. The random bred mice were typed by testing tail blood samples according to the GPI analysis method detailed in a subsequent section. Breeding stocks homozygous for the Gpi-1a allele and similar stocks homozygous for the Gpi-1b allele were established and maintained at Brock University.

Females in estrus were caged individually with males in the late afternoon. The mice were maintained on a 14 hour light, 10 hour dark cycle and mating was assumed to occur at the midpoint of the dark cycle, that is, around 12 a.m. Mating was detected by the presence of a vaginal plug, and the morning on which the plug was found was considered to be Day 0 of the pregnancy. The afternoon of the first day post coitum is therefore Day 0.5 of pregnancy.

Eight-cell embryos were obtained by flushing the oviducts in the late morning or early afternoon of Day 2 while blastocysts were obtained by flushing the uteri in the late morning or early afternoon of Day 3.

Maintenance of cell lines

The two EC cell lines used in this study were P10 and P19, and were a gift from M.W. McBurney. These EC cell lines were derived by him from the tumors induced following grafting of 7.5 day C3H embryos to the adult kidney or testis. P10 is a female, diploid, karyotypically normal cell line (McBurney and Strutt, 1980) while P19 is a male, diploid, karyotypically normal cell line (McBurney and Rogers, 1982). Both cell lines are homozygous for the Gpi-1b allele.

Frozen cell stocks of the cultures used in this study were suspended in fetal calf serum containing 10% DMSO and stored at -70°C. The P10 cells used were grown up from frozen stocks of originally diploid cells (karyotyped by M.W. McBurney). P10 is a feeder dependent cell line. STO cells (a mouse fibroblast cell line derived by A. Bernstein, and described by Martin and Evans, 1975) were used to establish a feeder layer. The STO cells were maintained in α-modified MEM (Gibco) containing penicillin and streptomycin, and supplemented with 5% newborn and 5% fetal calf serum (Flow Laboratories). Preparation of this medium is described in Appendix 1. The STO cells were subcultured every two or three days. When feeders were required, mitomycin C (Sigma) was added to confluent cultures to a final concentration of 0.01 mg/ml. The drug treatment was continued for 1.5 hr, then the cells were washed twice with PBS (Appendix 1) and harvested. The resuspended pellet from a confluent culture in a 60mm dish was used to establish feeder layers in three similar Falcon tissue culture grade petri dishes. Each dish was inoculated with about 1×10^6 feeder cells. Mitomycin C is an inhibitor of DNA synthesis which causes cross-linking of the DNA proportionally to the GC content (Harbers et al., 1968).

Dishes of feeders (freshly prepared, or not more than 2 days old) were inoculated with about 3×10^5 P10 cells. The EC cells grew slowly as colonies on top of the feeder monolayer, and were fed with fresh medium after 2 days and subcultured at a 1:4 dilution after 3 or 4 days. P10 cells will differentiate quite readily and continued proliferation of stem cells seems to be favored by this fairly frequent subculturing and vigorous trypsinization. The trypsin solution contained 0.125% trypsin (Sigma) in Ca^{++}, Mg^{++}- free PBS containing 0.2mM EDTA, and the colonies were dissociated in this solution by incubating at 37°C for about 5 minutes. After a few weeks in culture, the growth rate of the cells quite often increased. This increase in the growth rate may reflect the

loss of an X-chromosome (G. Paterno and M. McBurney, unpublished observations). While freshly thawed cultures were substituted quite frequently, it is possible that not all aggregations were made with XX cells.

The P19 cells used were from the P19S18 subclone. P19 cells are feeder independent, and were grown in α medium with very frequent subculturing, at fairly high cell densities. The cells were subcultured every 2 days, and sometimes daily, at dilutions no greater than 1:4 of a confluent culture (about 2.5×10^5 cells per dish) to maintain an exponentially growing culture of EC cells. Longer intervals led to extensive death of the stem cells and greater dilutions allowed emergence of a significant differentiated population.

P19S18O1A1 is a mutant clone of P19S18 selected for 6-thioguanine and oubain resistance (McBurney et al., 1982). It is a naturally occurring variant of P19S18 which will not incorporate [^{3}H]-hypoxanthine into DNA due to the lack of the enzyme hypoxanthine-guanine phosphoribosyl transferase. 6-thioguanine competes with guanine for HGPRT. As thio-GMP it is incorporated into nucleic acids as a poisonous analog (Harbers et al., 1968). This pathway is shown in Figure 6. These cells were also a gift from M. McBurney, and they behaved in culture like the P19S18 parental line.

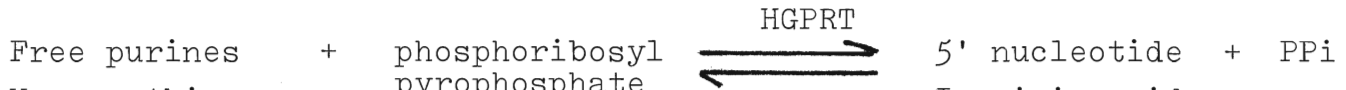
All cell cultures were grown at 37°C in a humid incubator containing 5% CO2 in air.

Preparation of EC-morula aggregates

Eight-cell embryos were flushed from the oviducts of 2.5 day pregnant mice in PB1 medium (Whittingham and Wales, 1969, Appendix 1). These embryos were mostly homozygous Gpi-1a/Gpi-1a except those used for in vitro experiments which were untyped. Mouth controlled finely drawn Pasteur pipets were used for all

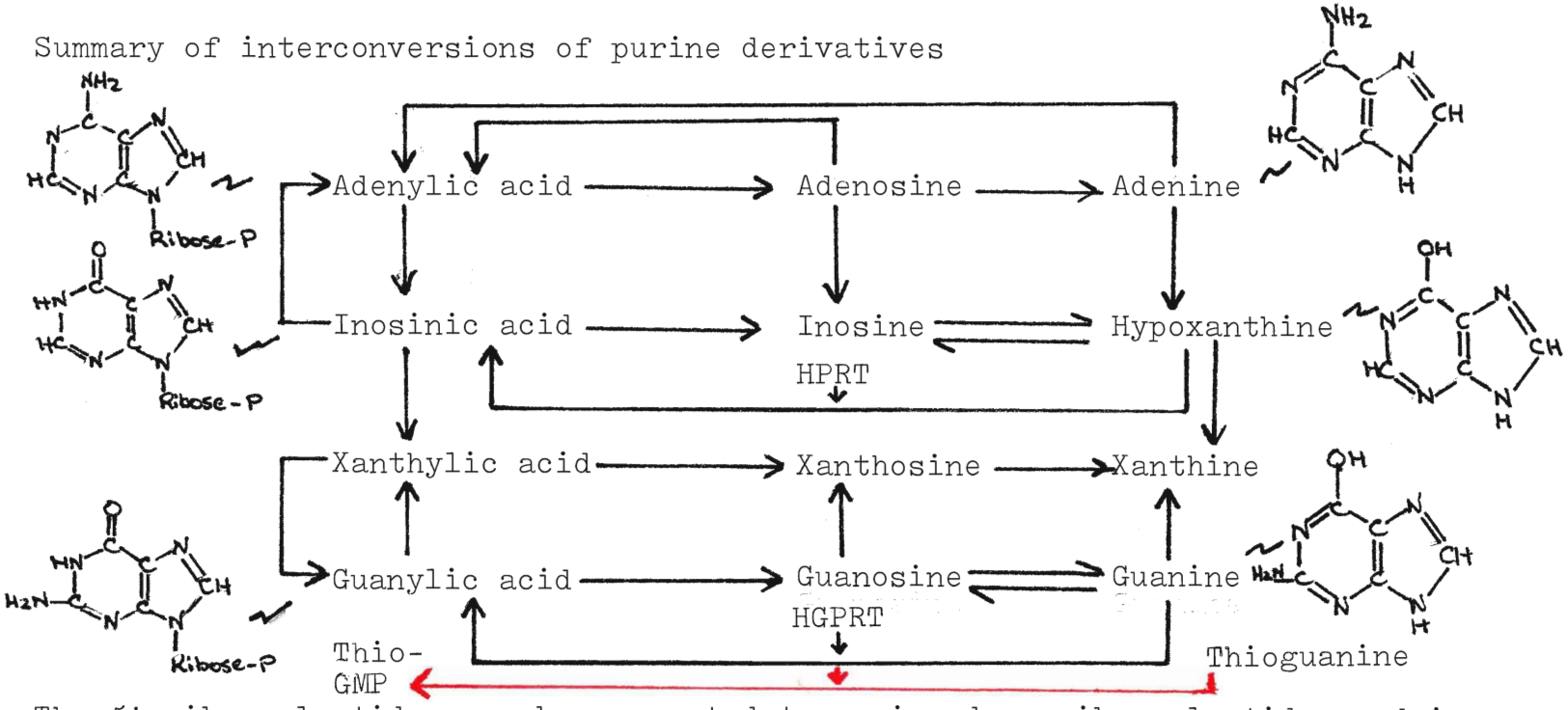
Figure 6 Interconversions of purine derivatives

The enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT or HPRT) is active in tissues in a salvage pathway, permitting reutilization of purines or purine derivatives from nucleic acid breakdown.



Inosinic acid is the precursor for both AMP and GMP.

Summary of interconversions of purine derivatives



The 5' ribonucleotides can be converted to purine deoxyribonucleotides and incorporated into DNA.

(from White et al., 1968)

flushing and embryo manipulations. The zonae pellucidae were removed by incubating the embryos for a few seconds in acid Tyrode's solution (Appendix 1) before transferring back to PB1 medium.

Aggregations were carried out in microdrops of α medium under oil. The embryos were washed in α medium then transferred singly to the microdrops.

The day prior to aggregations, a subconfluent EC culture was harvested and diluted about 1:4 into a bacteriological grade dish. EC cells adhere poorly to this surface, and remain in suspension. On the day of the experiment, EC clumps which resembled ICMs in size and morphology were selected for aggregation with the morulae. Feeder cells are larger than P10 cells and tend to attach even to this surface, and differentiated P10 cells tend to form very loose associations of rounded cells which were readily distinguishable from the EC clumps selected.

The EC-morula aggregates were made by gently blowing the EC cell clumps into contact with the embryos in the microdrops. The aggregates were incubated for about 30 minutes at 37°C in 5% CO2 in air then checked for adherence. Usually aggregation was beginning, but adjustments were made where necessary (Plate 2). The aggregates were then incubated overnight at 37°C in the CO2 incubator.

Isolation of inner cell masses by immunosurgery and preparation of ICM-morula aggregates

Homozygous Gpi-1b/Gpi-1b blastocysts were flushed from the uteri of 3.5 day pregnant mice in PB1 medium. Occasionally a few late morulae were recovered, but the embryos were usually at the early blastocyst stage. Zonae were removed with acid Tyrode's solution and ICMs were isolated by immunosurgery. Immunosurgery is a technique developed by D. Solter (Solter and Knowles, 1975)

which relies on the susceptibility of mouse blastocysts to complement-dependent antibody cytotoxicity. In the first step of the 2-step procedure, the blastocysts were incubated for 45 min at 37°C in a 1:8 dilution (in PB1) of a rabbit antiserum raised against mouse 12 day embryonic tissue by A. Croy. Unbound antibody was removed by rinsing the embryos twice in drops of PB1, then they were incubated in rabbit complement (Low-Tox M Rabbit Complement, Cedarlane Laboratories) diluted 1:10 in serum-free PB1, for 35 min at 37°C. During this incubation, complement mediated lysis of the trophoblast cells occurs, and cell debris can be removed by gentle pipetting. The ICMs were washed twice in PB1 and allowed to recover for at least 15 minutes in PB1 prior to aggregation.

Homozygous Gpi-1a/Gpi-1a morulae for aggregation with ICMs were collected in the same manner as for EC-morula aggregates, and transferred to α microdrops. The ICMs were rinsed in α medium then blown into contact with the morulae (Plate 2). Following adherence, these aggregates were also incubated overnight at 37°C in 5% CO2 in air.

Transfer of aggregates and control embryos to recipients

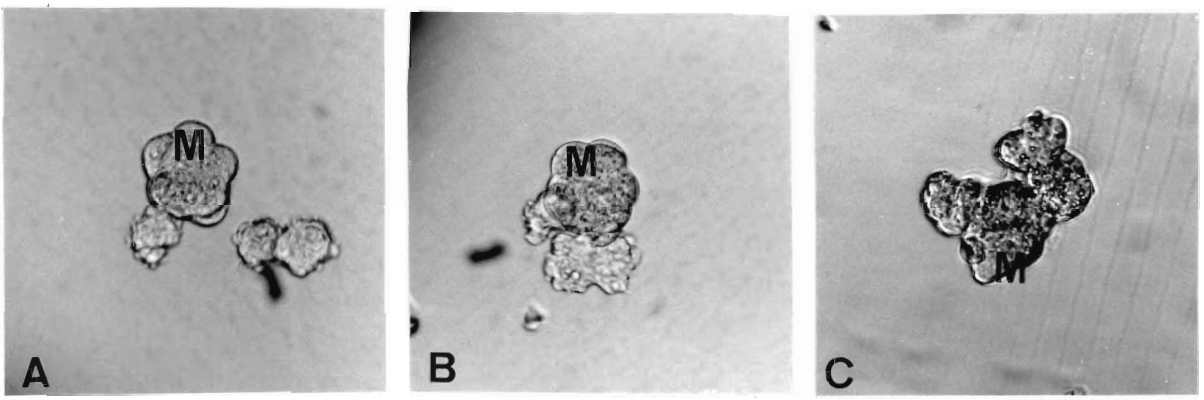
The following day, successful aggregates and controls were transferred to recipient foster mothers (homozygous Gpi-1a/Gpi-1a) which were 2.5 days pseudopregnant. Pseudopregnancy was established by mating females in estrus with vasectomized males. The physical stimulation of copulation induces the development of functional corpora lutea in the ovaries following ovulation, such that the uterus is prepared for implantation of embryos (Whitten and Champlin, 1978). Because the recipient is only 2.5 days pseudopregnant while 3.5 day embryos are transferred, some delay in development due to the experimental manipulations can be accommodated. From the time of transfer, gestational age was calculated from the recipient; that is, the day of transfer was counted as

Plate 2 The formation of aggregates between P10 clumps or ICMs and morulae

A Aggregation of three ICM-sized clumps of P10 cells with a 2.5 day morula (M). Two of the P10 clumps have failed to adhere to the morula initially.

B The same aggregate, 40 min later, after adjustment.

C Aggregation of three ICMs with a morula (M). The aggregate was photographed 45 min after construction.



50μm

Day 2.5 of pregnancy.

The mouse uterus is bicornuate, and five to six aggregates were transferred to one uterine horn of each recipient while controls were transferred to the contralateral horn. The controls were embryos which had been flushed on the previous day and treated in the same way as the morulae used in the aggregates.

The embryos were surgically transferred to the recipients (anaesthetized with Avertin, Appendix 1) through a dorsal incision in the body wall. The transfer pipet was a finely drawn Pasteur pipet filled partly with light paraffin oil. The embryos were drawn into the capillary stem in PB1 medium and delivered through a needle hole into the uterus in a minimal volume of PB1.

Blastocyst injections

Blastocyst injections were performed by Janet Rossant, using the technique described by Gardner (1968, 1978). The injections were made with a Leitz micromanipulator assembly, with the blastocysts in hanging drops of medium in manipulation chambers filled with heavy paraffin oil. The blastocysts were held on a holding pipet by suction at the embryonic pole and a triangular hole was made in the trophectoderm opposite the ICM with three glass needles. EC cells were injected through this hole into the blastocoelic cavity of homozygous Gpi-1a/Gpi-1a 3.5 day blastocysts via an injection pipet. Injected blastocysts were allowed to recover for about 1 hour before transfer to the recipient uteri.

Recovery of embryos at 9.5 to 11.5 days development from recipients

Taking the day of transfer as Day 2.5 of pregnancy, the transferred embryos were allowed to develop in utero for 7 to 9 days after transfer, and recovered between Day 9.5 and Day 11.5 of pregnancy. After careful assessment and

comparison of the developmental stage achieved in both control and experimental embryos, the experimental embryos were dissected into three component tissues, and the tissues were prepared for GPI analysis. Each conceptus was dissected into ectoplacental cone (EPC), yolk sac (extraembryonic membrane), and embryonic fractions. Each tissue sample was transferred to a clean petri dish in PBS, washed in PBS, and a drop of distilled water was added to each sample. The samples were then frozen at -20°C.

GPI analysis

Glucose phosphate isomerase (GPI) was used as the marker enzyme to monitor the contribution of the added EC cells or ICM cells to the developing chimeras. The method used for GPI analysis of the embryonic tissue samples recovered was the micro starch gel technique developed by A. Peterson (Peterson et al., 1978). This method is designed to detect GPI isozymes in nanogram tissue samples, and is sensitive enough to detect a 5% contribution by each isozyme in chimeric samples.

The gels were 12% electrostarch (Electrostarch Co., Wisconsin) in 3.75 mM Tris, 1.3 mM citrate buffer, pH 7.2. After heating the starch mixture in a stoppered side-arm flask over a flame until it cleared and thickened, the mixture was degassed by connection to an aspirator, and distributed amongst gel molds. The gels were allowed to set in the molds at room temperature for 2 hours, forming gels 8 cm x 10 cm x 2mm thick. The samples were adsorbed onto small pieces of cellulose polyacetate electrophoresis strips (Sepraphore III, Gelman) dipped in tissue (or blood) lysates. After blotting, the samples were inserted into small grooves cut in the gel with a gel comb, the gels were positioned across bridge tanks, wicks were applied, and the gels were covered with plastic film. Electrophoresis was at 250 volts for 1.5 hr at 4°C. The

running buffer was 0.13 M Tris, 0.047 M citrate, pH 7.2 and migration of the GPI isozymes was towards the cathode. Following separation of the isozymes, they were visualized by staining.

Nitrocellulose filters (Sartorius, 47mm diameter, 0.45μm pore size) were applied over the gels and the samples were allowed to absorb onto the filters for about 10 minutes. A staining well was created by greasing a rubber O-ring of slightly smaller diameter than the filter and pressing it firmly into place on the filter. The staining mixture for 4 filters contained the following:

7.6 ml Tris-citrate running buffer (0.13m Tris, 0.047M citrate, pH 7.2)

0.2 ml D-fructose-6-phosphate (Sigma, diNa salt, Grade 1, 75 mg/ml)

15μl glucose-6-phosphate dehydrogenase (Sigma, Type XV from Baker's yeast, 50 units/ml)

0.2 ml NADP (Sigma, Na salt, 10 mg/ml)

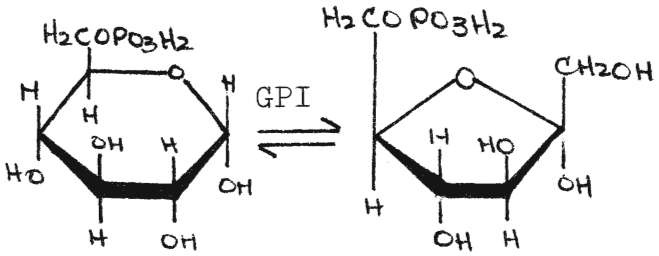
0.2 ml PMS (phenazine methosulfate, Sigma, 1.8 mg/ml)

0.2 ml NBT (nitro blue tetrazolium, Sigma, Grade III, 10 mg/ml)

1.3 ml MgCl2 (0.25M MgCl2)

Staining was carried out at 37°C in the dark for about 30 minutes. In the staining reaction, the formation of glucose-6-phosphate, catalyzed by the isomerase utilizing fructose-6-phosphate as the substrate, is followed by dehydrogenation catalyzed by glucose-6-phosphate dehydrogenase, and reduction of NADP. This reduction is coupled to the reduction of the tetrazolium dye, producing formazan which precipitates onto the filters as a blue spot wherever glucose phosphate isomerase is present. These reactions are shown in Figure 7.

Figure 7 The reactions in the staining procedure for glucose phosphate isomerase.

1. 

Glucose-6-phosphate Fructose-6-phosphate

2. Glucose-6-phosphate + NADP $\underset{\text{G-6-P dehydrogenase}}{\rightleftharpoons}$ 6-phosphogluconic acid + $NADPH_2$

3. $NADPH_2 + X \longrightarrow NADP + XH_2$ (X = reducible dye)

The morulae used in aggregations were homozygous Gpi-1a/Gpi-1a as were the recipients. The EC cells and the ICMs were homozygous Gpi-1b/Gpi-1b. Therefore, the appearance of the BB isozyme band in the embryonic tissue samples analysed was the indicator of chimerism. The stained filters were permanently preserved by submersion in H2O-ethanol-acetic acid (5:5:1) fixative overnight. After rinsing, the relative quantities of AA and BB isozymes in each chimeric sample were estimated visually from the densities of the blue spots.

Recovery of embryos on Day 5.5 or Day 6.5: Tissue fixing, processing and embedding for histology

In some experiments, development of embryos was assessed at an earlier stage, at the time of egg cylinder formation in normal development. At this time the embryo is very small and is embedded within a large maternal decidual swelling, and is best examined in histological preparations. Recipients were killed 3 or 4 days after the transfers, on Day 5.5 or Day 6.5, and the uteri were pinned onto foil supports for fixing overnight in AFA fixative (H2O:ethanol:40% formalin:acetic acid, 5:3:1:1). The following day, the AFA was poured off, 50% ethanol was added for 1 hour, followed by 70% ethanol for at least 1 hour, and the tissue was then ready for further processing in a Tissuematron automatic processor.

Each control or aggregate implantation site, easily recognizable as a prominent swelling, was dissected from the uterus and these samples were processed in the Tissuematron. In the automatic processing, they were first dehydrated through a series of alcohols, then cleared in xylene, and finally infiltrated with paraffin wax (TissuePrep, Fisher Scientific). This series is described in Appendix 1. Each implantation site was embedded in a wax block and serial 7μm sections were cut sagittal to the embryo on a rotary microtome. The

sections were mounted on slides with Mayer's albumin then stained with hematoxylin and eosin (Appendix 1) and cover-slipped with Canada balsam.

In vitro culture of embryos

In some experiments, the embryos were not transferred to recipients following the overnight culture period, but were allowed to continue their development in vitro in the microdrop cultures. The following day (48 hours after flushing the morulae) the embryos were transferred to a tissue culture grade surface in order to facilitate attachment and blastocyst outgrowth. These outgrowths were sometimes cultured in α microdrops in tissue culture petri dishes, but were more conveniently established in tissue culture chambered slides (8 chambers, Lab-Tek) with about 300 μl of medium in each chamber. After 4 or 5 days in the chambered slides, the outgrowths were fixed and stained. After pouring off the medium, each outgrowth was washed with PBS, fixed for about 10 minutes in ethanol-acetic acid (3:1), washed with ethanol, and allowed to air dry prior to staining with Giemsa (Appendix 1).

Labelling of outgrowths and autoradiography

In some experiments, a positive identification of the embryo derived cells in outgrowths from EC-morula aggregates was desirable. The embryo derived cells could be distinguished from EC derived cells if the aggregates were made with hypoxanthine phosphoribosyl transferase negative (HPRT-) P19S18O1A1 (O1A1) mutant cells. After the outgrowths were well established in chambered slides (usually 6 days to 1 week after the EC-morula aggregates were made) the cells were incubated with [^{3}H]-hypoxanthine in a pulse labelling. The culture medium was withdrawn from each well and 200 μl of fresh α medium, containing 2 μCi [^{3}H]-hypoxanthine (Amersham, 1.2 Ci/mmol) were added to each well. Labelling was

continued for 2 hours at 37°C in the CO2 incubator, then the medium was withdrawn, the outgrowths were washed with PBS, and fixed as before. In preparation for autoradiography, the partioning piece and the gasket were removed from each chambered slide, leaving a standard microscope slide. The slides were coated with Kodak NTB2 Nuclear Track Emulsion in a dipping chamber, then left in the dark, dry, at 4°C for 1 week before developing (Kodak D19 developer, 1.5 min; stop; fixer, 5 min) and staining with Giemsa.

Retinoic acid treatment of EC cells

In some experiments, P19 (P19S18 or P19S1801A1) cells were treated with retinoic acid (RA) prior to aggregation with morulae. The retinoic acid (all trans, Eastman) was dissolved in DMSO at 10^{-2} M and aliquots were stored at -70°C. These stocks were freshly prepared every 3 months. When treatment was to be initiated, an aliquot was serially diluted to 10^{-5} M in PBS. In 48 hr RA treatments, RA was added to a subconfluent monolayer to 10^{-7} M for the first 24 hr period. The cells were then harvested, diluted 1:4 into a bacteriological dish and fresh retinoic acid was added, again to 10^{-7} M. In 24 hr treatments, the retinoic acid was added to a freshly inoculated bacteriological dish to a final concentration again of 10^{-7} M RA. These suspension cultures were incubated overnight, and small clumps were selected the next day and used to make aggregates with morulae. These aggregates were cultured overnight with no further retinoic acid treatment, then transferred to recipients or cultured further in vitro. In some cases, treated P19 cells were injected into blastocysts.

Aliquots of RA-treated cell clumps were plated out along with aliquots of untreated cells from overnight suspension cultures into tissue culture dishes to monitor differentiation of the cells in vitro. Differentiated cell cultures

could be fixed and stained with Giemsa.

Indirect immunofluorescence assays

In some experiments, a positive identification of undifferentiated EC cells amongst the various cells in outgrowths from RA treated lumps was desirable. An indirect immunofluorescence assay was employed for this purpose. This assay takes advantage of the expression of SSEA-1 antigen by undifferentiated EC cells, and the loss of the stage specific embryonic antigen during differentiation (Solter and Knowles, 1978). P19S18O1A1 clumps, untreated, or pretreated with retinoic acid for either 24 or 48 hours, were plated out in tissue culture chambered slides. To facilitate adherence and spreading of the cells, small clumps, about the size of those aggregated with morulae or injected into blastocysts, were selected and about six to ten were added to each well. Following a 2 day outgrowth period, the medium was removed from each well, the cells were washed twice with PBS, then fixed with cold methanol for 10 minutes on ice and air dried. Each outgrowth was then incubated with normal rabbit serum, diluted 1:300 in 200μl of PBS for each well. This preincubation was designed to block any non-specific antibody binding in the subsequent reactions. Following this 30 min preincubation on ice, the outgrowths were rinsed 2X in PBS and incubated with the primary antibody. The primary antibody was monoclonal IgM anti-SSEA-1, a gift from D. Solter. The recommended dilution for this preparation was 1:200 to 1:300 and after trials with control P19 cultures, a dilution of 1:200 in 200μl PBS per well was used in all assays. After a 30 min incubation at 37°C, the outgrowths were washed 3X with PBS, and incubated with the secondary antibody. The secondary antibody was an anti-IgM antibody raised in a rabbit and FITC conjugated (that is, conjugated with fluorescein isothiocyanate). This was supplied as a lyophilized preparation from Bionetics

and reconstituted to 2 ml with sterile distilled water. Aliquots were stored at -70° C and a dilution of 1:10 in PBS was found to be appropriate after trials on control P19 cultures. After a 45 min incubation with the secondary antibody at 37°C, the outgrowths were washed 5X with PBS. The partioning piece and gasket were removed from the chambered slides and the outgrowths were mounted in glycerol-PBS (9:1) and examined for fluorescence on a Zeiss Universal photomicroscope equipped with epi-fluorescence blue (485) and green (546) filters.

Fluorescence was photographed on Kodak Tri-X (ASA 400) film, using a constant exposure of 120 sec, at a reciprocity setting of 0 on the Zeiss MC 63 exposure meter.

Karyotyping procedures: Chromosome preparations from P10 cells

Chromosome preparations were made from the P10 cells using the methods of McBurney (1976) as a guide. A well developed P10 culture was split 1:2 the day previous to provide an exponentially growing culture. Colcemid (Gibco, 10 μg/ml) was added to each culture to a final concentration of 0.06 μg/ml and the cultures were incubated at 37°C for 15 min. The medium was withdrawn and collected along with a PBS wash to minimize the loss of floating mitotic cells, and the remaining cells were harvested by trypsininzing. The total cell pellet was resuspended in about 1 ml of medium. Following swelling in hypotonic (0.56%) KCl for 10 to 15 min at room temperature, the cells were spun down and resuspended in a minimal amount of KCl. The cells were fixed for 30 min on ice in methanol:acetic acid (3:1) added dropwise initially to 10 ml. The cells were next spun down and resuspended in a small volume (about 1 to 1.5 ml) of cold fix. Slides were dipped in ice-cold water, then the cell suspension was dropped from a 15 cm height onto a wet slide and the spread was dried quickly over a

flame. The spreads were stained with Giemsa in phosphate buffer, pH 6.8 (Appendix 1) or alternatively, some spreads were G-banded, according to the following procedure. The slides were rinsed in 0.15 N NaCl then dipped in 0.1% trypsin in 0.15N NaCl for 20 to 30 sec. They were then rinsed 2X in 0.15 N NaCl, stained for 6 min in Giemsa in phosphate buffer, pH 6.8, rinsed 2X in distilled water and dried.

RESULTS

I AGGREGATIONS OF EC CELLS WITH MORULAE

1. Aggregations of P10 cells with morulae

1a. Aggregations of a single P10 clump with a morula

In these experiments single clumps of P10 cells which resembled ICMs in size and morphology were selected from suspension cultures and aggregated with morulae. Clumps of this size contain about 15 to 20 EC cells (Rossant and Papaioannou, in press). Transferred embryos and controls were left to develop for seven to nine days in the recipients following transfer and were recovered on Day 9 to Day 11 of development. Table 2 gives embryo recovery data for these experiments.

The implantation and embryo recovery rates were high for both controls and aggregates in these experiments. The aggregate embryos recovered are described in Table3. The first six embryos in this table were recovered at 9.5 days gestation while the next 16 were recovered at 10.5 or 11.5 days gestation. GPI analysis indicated that all 22 embryos were chimeric, and that P10 contribution to these embryos was very high. Six of these embryos (numbers 9, 12, 13, 15, 18 and 21) were judged to be totally P10 derived and a further ten embryos were considered to be at least 75% P10 derived. In only one embryo (#4) was the P10 contribution judged to be less than 50%.

These embryos were almost all judged to be developing abnormally at midgestation when compared to controls. The control fetuses were all well-formed. The head was always well developed, with visible optic vesicles and full closure of the neural tube evident. Body development had reached the limb bud stage, and there was an extensive array of somites.

Table 2

Embryo recovery data following transfer of P10-morula aggregates and controls

	Control morulae	P10-morula aggregates
embryos transferred	24	41
implants (% transferred)	19 (79%)	29 (71%)
resorptions (% implants)	4 (21%)	7 (24%)
embryos (% implants)	15 (79%)	22 (76%)

overall embryo recovery:

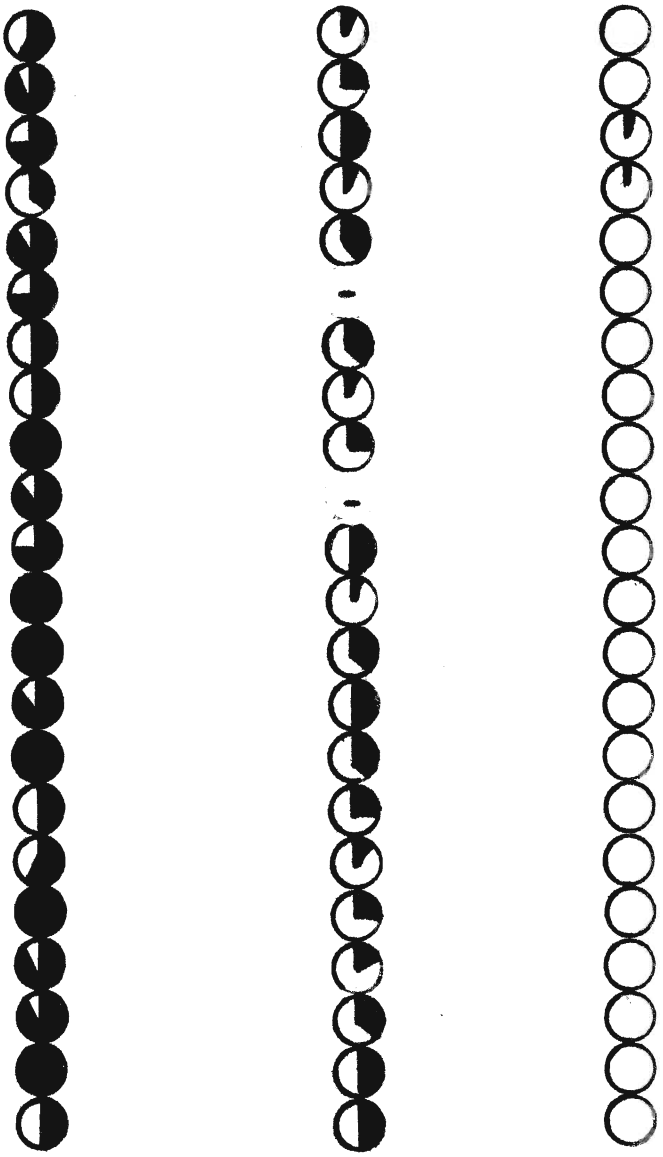
embryos recovered/embryos transferred:

controls: 62% aggregates: 54%

Table 3

Aggregations of a single P10 clump with a morula

Embryo and description	GPI Analysis		
	Embryo proper	Extraembryonic membranes	EPC
1. small, allantois unfused			
2. small, allantois unfused			
3. small			
4. normal			
5. small, allantois unfused			
6. very small			
7. very small, little body			
8. misshapen head, little body			
9. small, little body			
10. small, swollen, allantois unfused			
11. disorganised tissue			
12. small, swollen, allantois unfused			
13. misshapen head, little body			
14. disorganised tissue			
15. swollen head, little body			
16. small, deformed			
17. small, allantois unfused			
18. small, allantois unfused			
19. swollen head, allantois unfused			
20. swollen head, allantois unfused			
21. very small			
22. extremely small			



EPC: ectoplacental cone fraction of the conceptus

The black portion of each diagram represents P10 contribution. The first 6 embryos were recovered at 9.5 days gestation, while the remainder were recovered at 10.5 to 11.5 days gestation.

The P10 chimeras on the other hand frequently were small and retarded in their development relative to the controls. Typically, they had a misshapen head, sometimes larger on one side than the other, and the neural tube was often open. The head often had a swollen appearance. These embryos generally exhibited very little body development, and an array of somites was very rarely seen. A common defect was an unfused allantois, recognized as a sac or bulb-like structure posterior to the embryo. Embryos 11 and 14 consisted of disorganized tissue, with the only recognizable structure being a small group of somites within an otherwise unformed sac of tissue in Embryo #14. Embryo #4 was the only embryo judged to be developing normally. This was the embryo rated as having the lowest P10 contribution, around 40%.

The P10 cells also contributed to the extraembryonic membrane fraction of these chimeras, but the P10 contribution to this fraction was considerably less extensive than to the embryo proper. Contribution was estimated at 50% in five samples, and less than 50% in the remainder.

The P10 cells did not contribute to the ectoplacental cone fraction of the chimeric conceptuses except for a trace in two samples.

1b. Aggregations of three P10 clumps with a morula

In this series of P10 aggregations, the P10 component of each aggregate was increased by aggregating three of the ICM-sized clumps of EC cells from suspension cultures with each morula. Table 4 shows the embryo recovery data for this series.

Table 4 Embryo recovery data following transfer of triple P10-morula aggregates and controls

	Control morulae	3P10-morula aggregates
embryos transferred	39	31
implants (% transferred)	35 (90%)	23 (74%)
resorptions (% implants)	6 (17%)	6 (26%)
embryos (% implants)	29 (83%)	17 (74%)

overall embryo recovery:

embryos recovered/embryos transferred

controls: 74% 3P10-morula aggregates: 55%

Again, these aggregate embryos implanted in the uterus at a high rate following transfer and the resorption rate in the early post-implantation period was low such that embryo recovery was high at midgestation. Table 5 describes 15 of the 17 aggregate embryos recovered in these experiments (two embryos recovered were too small for GPI analysis). Again, GPI analysis revealed that all 15 embryos were chimeric. However, P10 contribution to the first ten embryos was generally lower than that seen in the previous set. None of these embryos was judged to be totally P10 derived, and only four of the ten were judged to at least 75% P10 derived. The remaining six embryos had a maximum of 50% P10 contribution. These first ten embryos were recovered at 9.5 days gestation. These embryos were very small and undeveloped compared to the controls, and were smaller than the comparable single P10-morula aggregates recovered at 9.5 days. The next five embryos were allowed to develop a further two days before recovery at 11.5 days gestation. These five embryos had very high P10 contributions, and were all judged to be totally, or very nearly totally P10 derived.

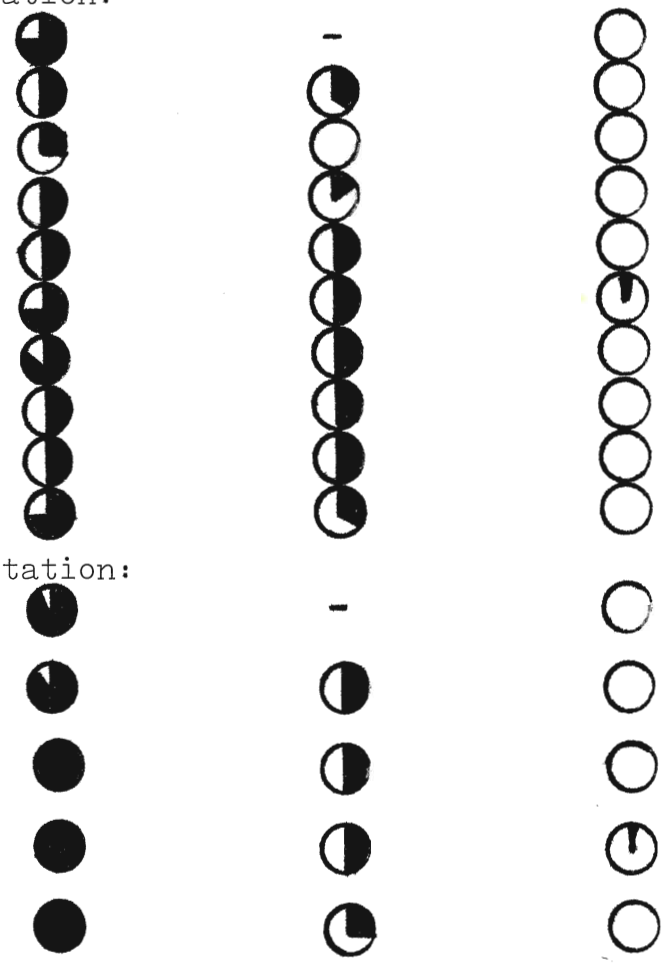
The embryos recovered at 9.5 days were so retarded in their development that specific abnormalities could not be assessed. By 11.5 days development abnormalities were clearly evident. Typically, the head was misshapen, the body showed very little form, and the allantois was unfused. Control embryos recovered in these experiments were all developing normally.

Again, P10 contribution to the extraembryonic membranes was lower than to the embryo proper, and P10 contribution to this fraction did not increase with continued development. Although no membrane fraction had a P10 contribution greater than 50%, eight of 13 fractions analysed were judged to be 50% P10 derived. In the previous set, where P10 contribution to the aggregate was lower at the outset, only five of 20 membrane fractions were judged to be at least 50%

TABLE 5

Aggregations of 3 P10 clumps with a morula

Embryo and description	GPI Analysis		
	Embryo proper	Extraembryonic membranes	EPC
Embryos recovered at 9 days gestation:			
1. small		-	
2. undeveloped body			
3. small			
4. small			
5. small			
6. small			
7. small			
8. swollen, disorganized			
9. very small			
10. very small			
Embryos recovered at 11 days gestation:			
11. extremely small, badly formed		-	
12. larger, misshapen head, little body development			
13. misshapen head, unfused allantois			
14. misshapen head, little body development			
15. misshapen head, unfused allantois			



EPC: ectoplacental cone fraction of the conceptus

The black portion of each diagram represents P10 contribution.

derived.

The P10 cells again did not colonize the ectoplacental cone fraction of these conceptuses beyond a trace in two samples.

1c. Aggregations of a single P10 clump with two morulae

In this series of P10 aggregations, the P10 component of each aggregate was decreased at the outset by aggregating a single P10 clump, containing 15 to 20 P10 cells, with two early morulae. Therefore, in these aggregates the numbers of EC cells and normal embryonic cells were about equal. These aggregates were transferred to recipients with the appropriate controls, which in these experiments were double morula aggregates. Recovery of these embryos was undertaken at 10.5 days development and the embryo recovery data is given in Table 6.

Embryos from both control and double morula-P10 aggregates were recovered at a reasonable rate in these aggregations also. The double morula control embryos were all developing normally and five of these ten control embryos appeared to be equivalent to embryos developing from transferred single morulae at 10.5 days development. The neural tube was fully closed and body development had reached the limb bud stage, with an extensive array of somites. In the other five control embryos, some developmental retardation was noted, but all were properly formed and further along than retarded P10 aggregate embryos. The double morula-P10 aggregate embryos are described in Table 7.

Table 6 Embryo recovery data following transfer of double morula-P10 aggregates and double morula aggregate controls

	double morula control aggregates	double morula-P10 aggregates
embryos transferred	16	24
implants (% transferred)	10 (62.5%)	18 (75%)
resorptions (% implants)	0	7 (39%)
embryos (% implants)	10 (100%)	11 (61%)

overall embryo recovery:

embryos recovered/embryos transferred

controls: 62.5% double morula-P10 aggregates: 46%

TABLE 7

Aggregations of a single P10 clump with 2 morulae

Embryo and description	GPI Analysis		
	Embryo proper	Extraembryonic membranes	EPC
1. misshapen head, little body development			
2. very small, badly formed			
3. badly formed, unfused allantois			
4. very small, badly formed			
5. neural tube open, unfused allantois, body development better			
6. small, neural tube open, unfused allantois, body development better			
7. small, neural tube open, unfused allantois			
8. small, better tube closure, poorer body development			
9. no embryo, growth of tissue on yolk sac			
10. small, better tube closure, poorer body development, unfused allantois			
11. no embryo in yolk sac			

e + ys

EPC: ectoplacental cone fraction of the conceptus

The black portion of each diagram represents P10 contribution.

GPI analysis indicated that again all the embryos recovered were chimeric, and despite the relatively lower P10 component of the aggregates initially, P10 contribution was very high by midgestation in each embryo. All the embryos had P10 contributions greater than 50%, and only one was judged to be less than 75% P10 derived. The other embryos were totally, or nearly totally P10 derived. In two cases, embryos and membranes were inseparable, but the remaining nine cleanly dissected membrane fractions were all chimeric and demonstrated a range of P10 contribution similar to that seen in the aggregates of a P10 clump with a single morula; that is, a marked decrease in P10 contribution to this fraction was not evident. The P10 cells did not colonize the ectoplacental cone fraction of any conceptus.

These P10 chimeras were also badly formed, with the abnormalities generally following the trends seen previously. In three cases, body development was better than that usually seen, with limb buds and somites present, but in two other cases a recognizable embryo was not present.

1d. Karyotyping of P10 cells

Chromosome preparations were made from P10 cells which had been established in culture from frozen stocks from M. McBurney, and maintained in this laboratory for two months. This karyotype analysis was undertaken at the completion of this project. A total of 38 metaphase spreads were examined, and the chromosome count was found to vary between 39 (20 spreads) and 40 (18 spreads). Obvious abnormalities such as major deletions or translocations were not noted. Analysis of one G-banded spread containing 39 chromosomes revealed that a very large chromosome lacked a mate. The banding pattern in this chromosome matched the banding in the X chromosomes in the karyotype analyses published by Robertson, et al. (1983) and this spread was assigned an XO

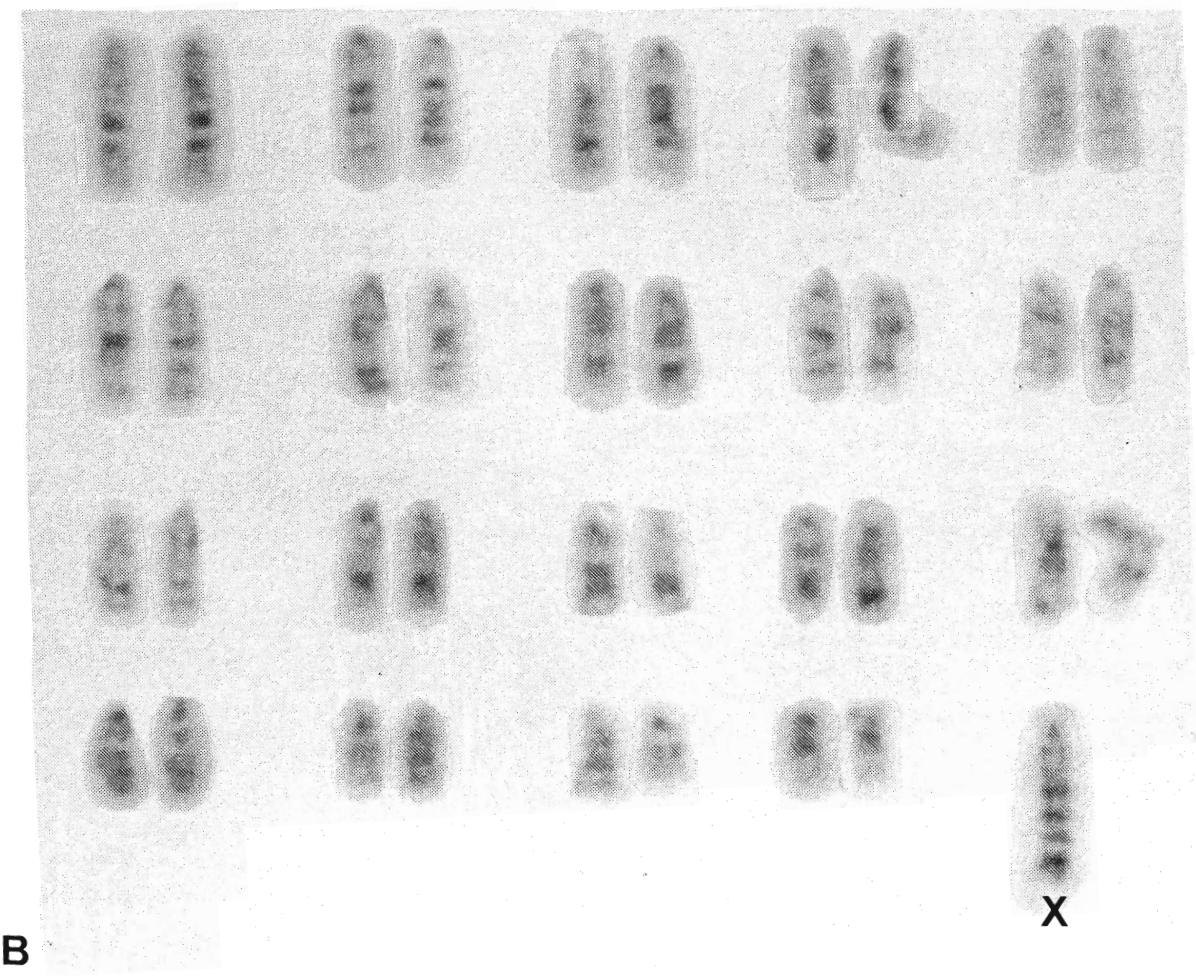
Plate 3 Chromosome preparations from P10 cells

An example of an XO genotype.

A A G-banded spread containing 39 chromosomes.

B These chromosomes were enlarged for a karyotype analysis. The large chromosome identified as an X chromosome had no mate.

A



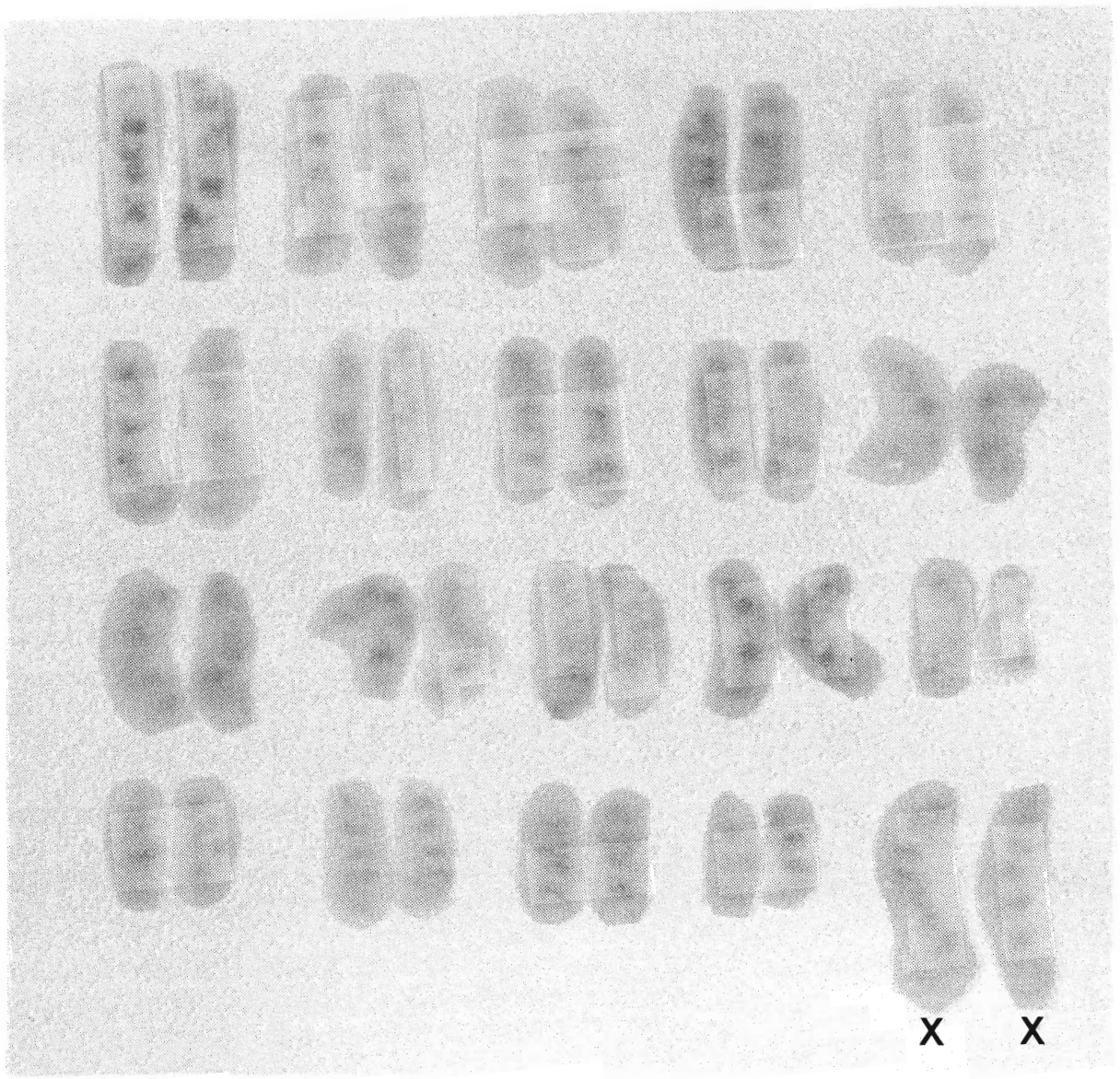
B

Plate 4 Chromosome preparation from P10 cells

A A G-banded spread containing 40 chromosomes.

B These chromosomes were enlarged for a karyotype analysis. Although the banding is faint, each chromosome appears to have mate.

A



B

genotype. Analysis of a spread containing 40 chromosomes suggested that two X chromosomes were present (plates 3 and 4).

2. Aggregations of P19 cells with morulae

2a. Aggregations of a P19 clump with a morula

In these experiments, P19 clumps were aggregated with morulae and the outcome was compared to P10 aggregations. An initial difference was noted in that P19 clumps were less "sticky" than P10 clumps from suspension cultures and were more difficult to aggregate with morulae. However, with several adjustments to the aggregates, better than 80% of these attempts were eventually successful, as were P10 aggregation attempts. The following day, P10-morula aggregates appeared to be well integrated morulae, in which the P10 clump could not be distinguished. The P19-morula aggregates did not generally appear to be so well integrated, and the P19 clump could usually still be recognized.

A more dramatic difference was noted following transfer of P19-morula aggregates to recipients. Embryo recovery data at 10.5 days development is given in Table 8 and compared to P10-morula aggregate embryo recovery at a similar stage.

While the P19-morula aggregates implanted in the uterus at a comparable rate to P10-morula aggregates, a very high percentage of these aggregates resorbed early such that embryo recovery at 10.5 days development was very low. The six embryos which were recovered are described in Table 9. These embryos were all judged to be developing normally, and GPI analysis revealed that these embryos were not chimeric. The P19 cells did not contribute to any fraction of the conceptus.

Table 8 Comparison of P19-morula aggregate embryo recovery to P10-morula aggregate embryo recovery

	P10-morula aggregates	P19-morula aggregates
embryos transferred	41	48
implants (% transferred)	29 (71%)	33 (69%)
resorptions (% implants)	7 (24%)	27 (82%)
embryos (5 implants)	22 (76%)	6 (18%)

overall embryo recovery:

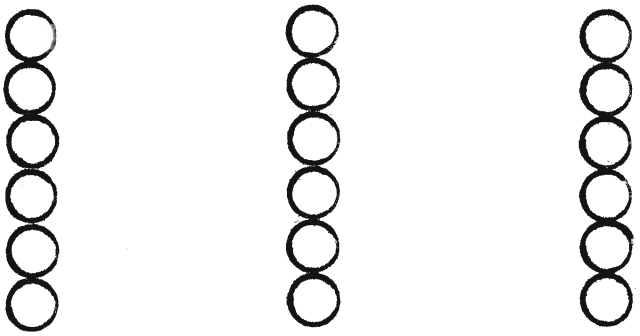
embryos recovered/embryos transferred

P10-morula aggregates: 54% P19-morula aggregates: 12.5%

TABLE 9

Aggregations of a single P19 clump with a morula

Embryo and description	GPI Analysis		
	Embryo proper	Extraembryonic membranes	EPC
1. Normal			
2. Small, but normal			
3. Small, but normal			
4. Small, but normal			
5. Normal			
6. Normal			



2b. Aggregations of retinoic acid pretreated P19 cells with morulae

In the first experiments of this type, the P19 cells were pretreated for 24 hours with retinoic acid in a monolayer culture, then for an additional 24 hours in a suspension culture. The P19 clumps in these cultures were not the well-integrated cell aggregates resembling ICMs which EC cells usually form in suspension culture. Instead, these cell clumps appeared to have cells on the surface which were less well integrated with the rest of the clump. These P19 clumps treated for a total of 48 hours with retinoic acid did not aggregate well with morulae. In 32 aggregation attempts, in two experiments, only five P19-morula aggregations were judged successful the following day. When RA pretreatment was decreased to 24 hours (in suspension culture), the P19 clumps from the suspension cultures were "sticky" and could be aggregated with morulae much more readily than those which had received the longer treatment. Embryo recovery data at 10.5 days gestation following transfer of these 24 hr RA+ P19-morula aggregates is compared to recovery of the untreated P19-morula aggregates at a similar stage in Table 10.

A better rate of embryo recovery was seen following pretreatment of the P19 cells with retinoic acid, but the early resorption rate was still very high, at 64%. The nine embryos recovered following treatment of the P19 cells with retinoic acid are described in Table 11. All these embryos were judged to be developing normally, and again GPI analysis indicated that these embryos were not chimeric.

Table 10 Comparison of untreated P19-morula aggregate embryo recovery to 24 hr RA+ P19-morula aggregate embryo recovery

	untreated P19-morula aggregates	24 hr RA+ P19-morula aggregates
embryos transferred	48	29
implants (% transferred)	33 (69%)	25 (86%)
resorptions (% implants)	27 (82%)	16 (64%)
embryos (% implants)	6 (18%)	9 (36%)

overall embryo recovery:

embryos recovered/embryos transferred

untreated P19-morula aggregates: 12.5%

24 hr RA+ P19-morula aggregates: 31%

TABLE 11

Aggregations of P19 clumps treated 24 hours with retinoic acid with morulae

Embryo and description	GPI Analysis		
	Embryo proper	Extraembryonic membranes	EPC
1. Normal			
2. Normal			
3. Slightly small, but normal			
4. Slightly small, but normal			
5. Slightly small, but normal			
6. Small, but normal			
7. Small, but normal			
8. Small, but normal			
9. Small, but normal			

2c. Injections of P19 cells treated 48 hours with retinoic acid into blastocysts

Since P19 cell clumps treated 48 hr with retinoic acid could not be aggregated with morulae, they were injected into blastocysts. The embryo recovery data at 9.5 to 14.5 days gestation and the descriptions of the embryos recovered following the injections are given in Table 12.

Embryo recovery was very high following injection of the treated P19 cells into blastocysts, and the great majority of these embryos were developing normally at midgestation and beyond. Again, the retinoic acid pretreated P19 cells had not contributed to any fraction of any conceptus.

2d. Cells outgrowing from P19 clumps following RA treatment

In order to monitor <u>in vitro</u> the effects of 24 hr and 48 hr retinoic acid treatments on the P19 cells, P19 cell clumps were plated out from treated and untreated suspension cultures. Untreated P19 cell clumps formed colonies of EC cells. At the periphery of these a few large flat cells were observed by the second day of culture. These differentiated cells morphologically resembled extraembryonic endoderm cells. The dense EC colonies began to degenerate quite rapidly after two days in culture, while the differentiated cells continued to proliferate.

When clumps of P19 cells treated with retinoic acid for 24 or 48 hr were plated out from suspension cultures, a much greater degree of differentiation was evident by the second day. The dense central areas of the colonies may have harbored undifferentiated EC cells but it was difficult to make a positive identification of EC cells by their morphology alone (plates 5 and 6).

The clumps plated out in the above experiments were a heterogeneous mixture of sizes. In subsequent experiments, small clumps, the size of those aggregated with morulae or injected into blastocysts, were selected from the suspension

Table 12 Embryo recovery following injection of P19 cell clumps treated 48 hr with retinoic acid into blastocysts

injected blastocysts transferred	implants (% transferred)	resorptions (% implants)	embryos (% implants)
36	32 (89%)	3 (9%)	29 (91%)

Embryo descriptions:

27 normal embryos

1 retarded embryo on the yolk sac

1 empty yolk sac

GPI Analysis:

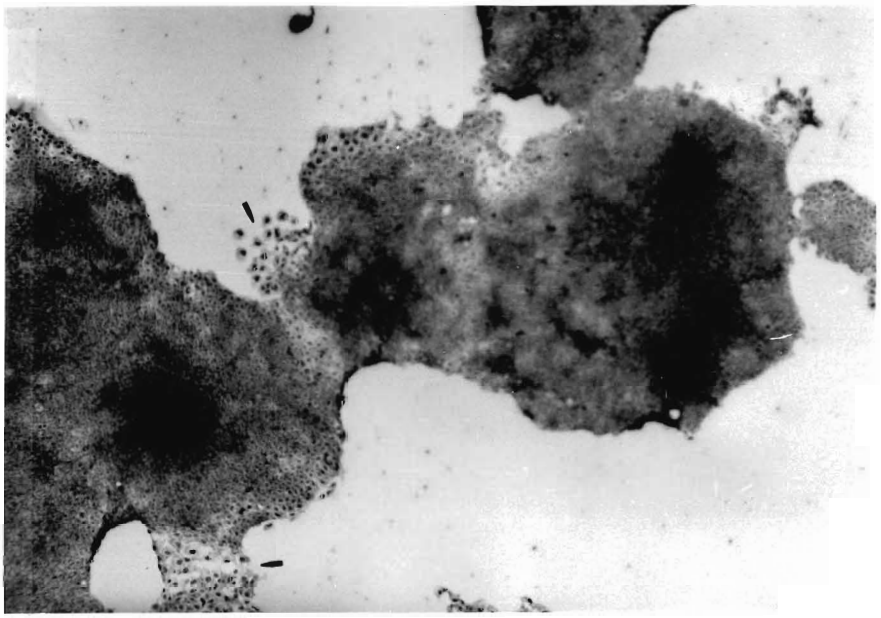
ALL EMBRYOS

NON-CHIMERIC

Plate 5 Retinoic acid induces differentiation of P19 cells *in vitro*

A An untreated culture of P19 cells, plated out after 24 hr in suspension culture, and stained 2 days after plating. The culture consists primarily of EC colonies, although spontaneously differentiated cells (arrows) can be seen at the peripheries.

B P19 cells plated out after 48 hr retinoic acid treatment. A high level of differentiation is evident within only 24 hr after plating.



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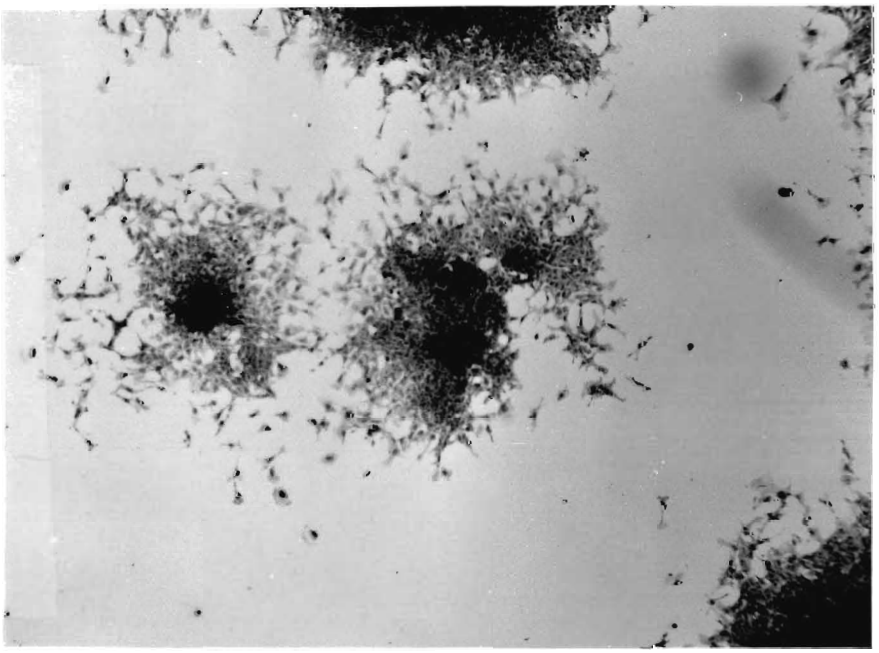
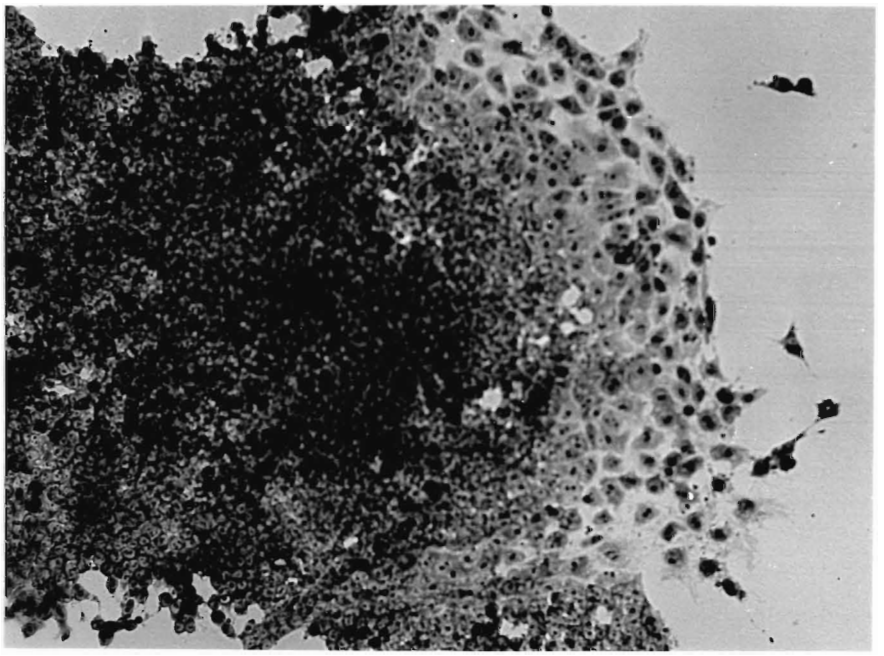


B

Plate 6 Undifferentiated EC cells may persist despite retinoic acid pretreatment.

A An untreated P19 culture, plated out after 24 hr in suspension culture and stained 2 days after plating. Higher magnification of a colony with differentiated cells at the periphery.

B P19 cells treated 24 hr with retinoic acid and stained 2 days after plating from suspension culture. While extensive differentiation is evident, EC cells might persist in the dense central regions of the colonies.



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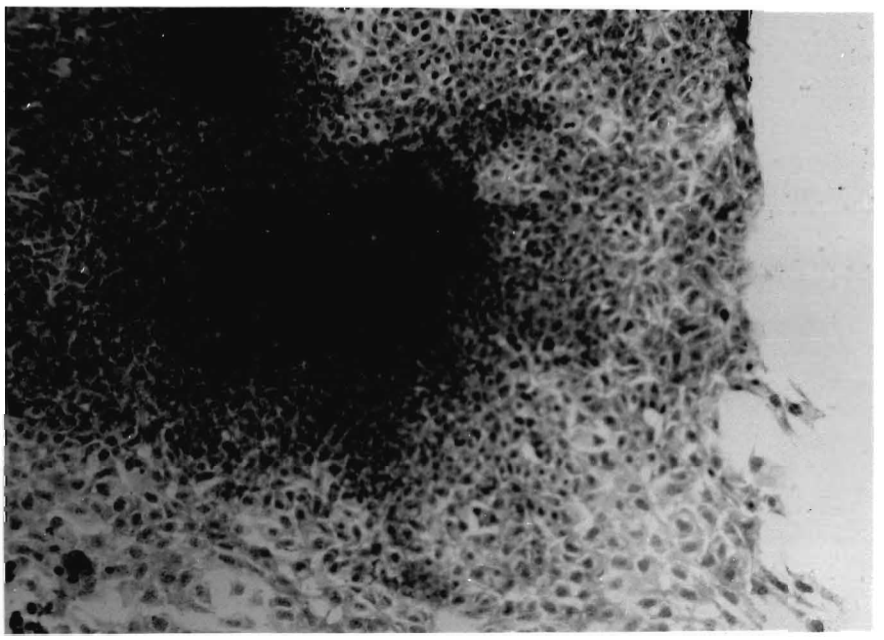


B

cultures and plated out. When small, untreated lumps were plated out, undifferentiated EC cells were the only cells present in most outgrowths after two days. When small RA treated clumps were plated out, a high level of differentiation was evident in these outgrowths by Day 2. However, cells with an EC-like morphology were clearly present in most. In 19 outgrowths from these small P19 clumps treated 48 hr with retinoic acid, 18 contained a small central focus of EC-like cells, surrounded by differentiated cells of an unknown type (Plate 7).

2e. Immunofluorescence assays

These experiments were designed to positively identify undifferentiated EC cells persisting in RA treated P19S18O1A1 cultures. When small clumps of untreated O1A1 cells were plated out from suspension cultures, the colonies appeared EC-like after two days, and fluorescence of entire colonies was observed. Following a 24 hr or 48 hr retinoic acid pretreatment, a high level of differentiation was evident in Day 2 outgrowths from small clumps. But clusters of cells with an EC-like morphology could be identified within these outgrowths, and these cells fluoresced, while the cells with a differentiated morphology did not (Plate 8). These EC-like cells, and the untreated cultures did not
fluoresce in the absence of anti-SSEA-1 in controls to which only the fluorescein tagged anti-IgM antibody was added.

2f. <u>In vitro</u> outgrowths of untreated or RA treated P19-morula aggregates

These experiments differ from those just described in that the untreated or RA treated P19 cells were aggregated with morulae and the aggregate embryos were

outgrown in vitro. P19S18O1A1 (O1A1) cells, which are hypoxanthine phosphoribosyl transferase negative (HPRT-), were used such that embryo derived cells in the aggregate outgrowths could be positively identified as the labelled cells in autoradiograms.

When untreated O1A1-morula aggregates were outgrown and labelled with [^{3}H]hypoxanthine, EC-like cells were usually, but not always, present. Most commonly, a heavy growth of EC-like cells predominated (Plate 9). These cells were unlabelled in the autoradiograms, as were other cells with a differentiated morphology present in these outgrowths. Labelled giant cells could be found, and occasionally a patch of labelling could be seen within the EC-like growth. This was the presumptive ICM of the embryo. In six such autoradiograms, five matched the above description, while in one, no cells with an EC-like morphology were seen. In this outgrowth, only a group of undifferentiated, unlabelled cells were present, off to the side of a normal appearing blastocyst outgrowth.

When the O1A1 cells were pre-treated for 24 hr with retinoic acid prior to aggregation with morulae and outgrowth, the unlabelled cells with the EC-like morphology were observed again in most outgrowths. In seven such outgrowths, four contained a growth of EC-like cells which usually appeared to be integrated with the embryonic ICM. The unlabelled differentiated cells present in these outgrowths usually appeared scattered, and not associated with the ICM and EC-like cells. In one other outgrowth, only these differentiated cells were present in addition to the embryonic cells, and in two others no EC or EC derived cells could be seen. These appeared to be normal blastocyst outgrowths.

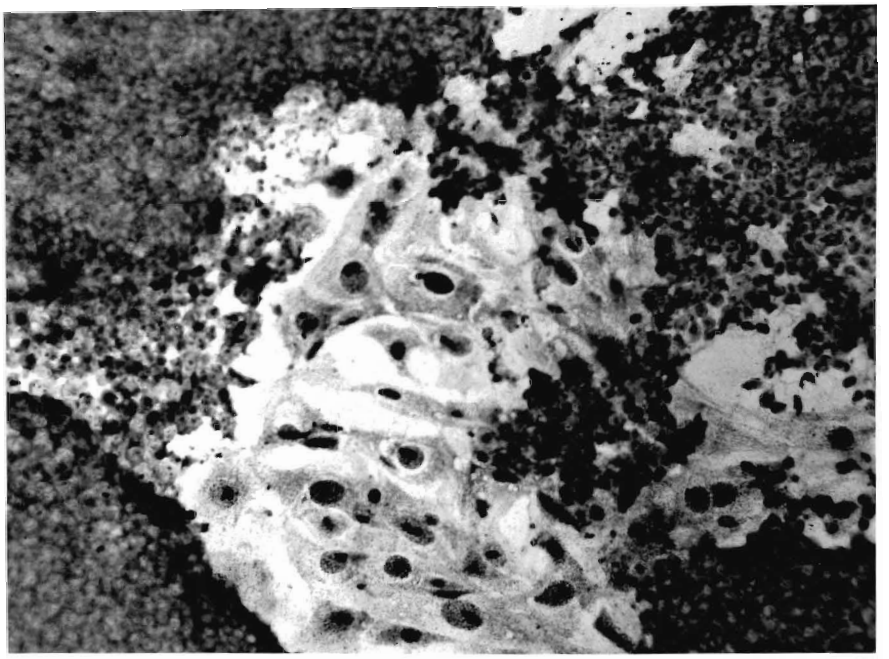
The <u>in vivo</u> and <u>in vitro</u> P19 results are summarized in Tables 13a and 13b. In Table 13b, the numbers refer to outgrowths established in chambered

slides. Additionally, cells were plated out from suspension cultures into petri dishes in all experiments involving retinoic acid treatment, and EC like cells were always seen.

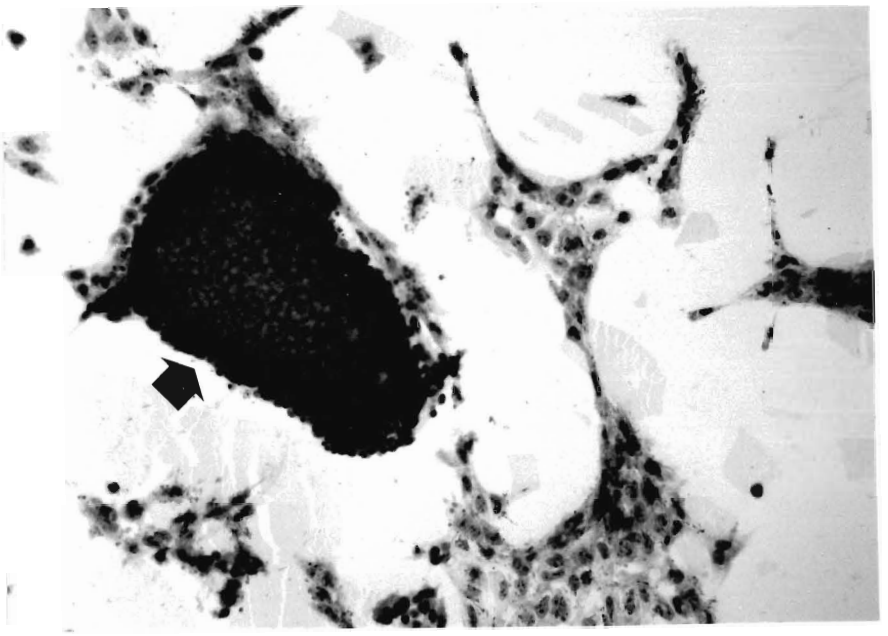
Plate 7 Further evidence that EC-like cells persist despite retinoic acid treatment

A A blastocyst outgrowth from a 24 hr RA+ P19(01A1)-morula aggregate. The embryonic trophectodermal giant cells (central) are surrounded by a massive growth of EC-like cells, which were not labelled in autoradiograms, and therefore were not of embryonic origin.

B Foci of EC-like cells (arrow) could be found in most outgrowths from small clumps of P19 cells (the size of those aggregated with morulae or injected into blastocysts) even after a 48 hr pretreatment with retinoic acid.



A



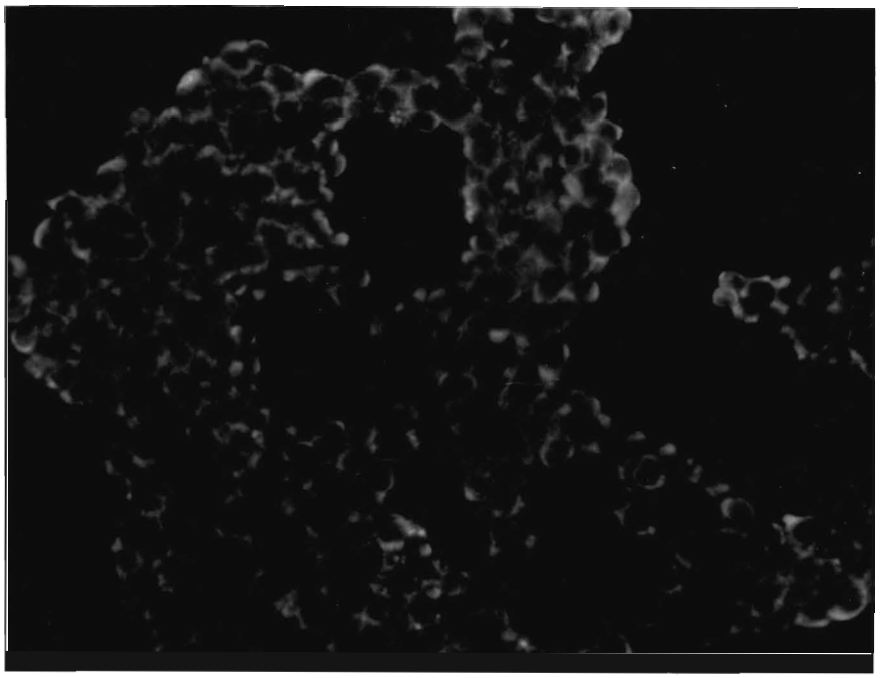
B

Plate 8 Positive identification of undifferentiated EC cells in retinoic acid treated P19 cultures

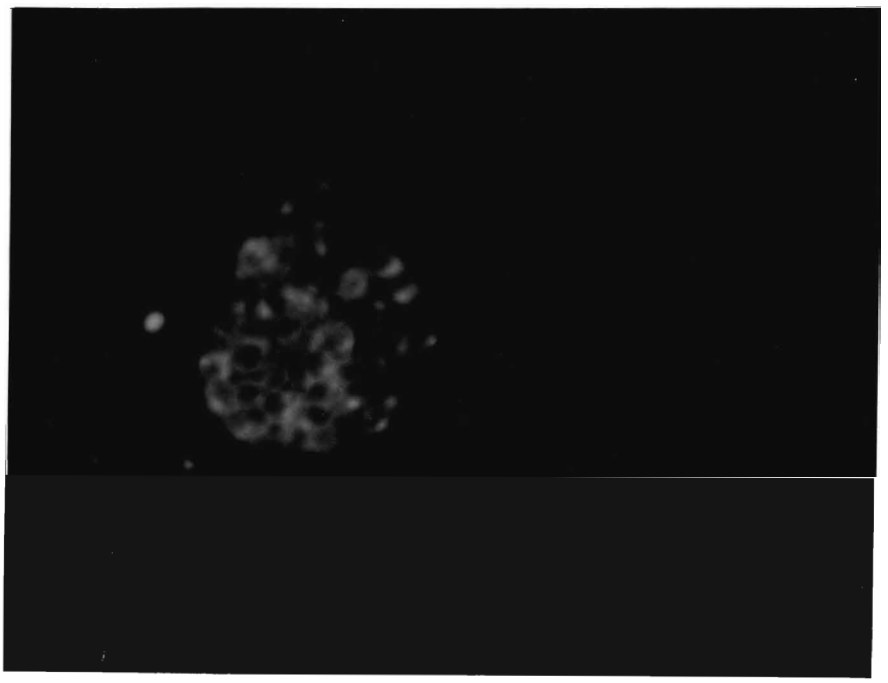
A P19 cells express SSEA-1. Fluorescence of an entire colony of untreated P19 cells, with an EC-like morphology, in an indirect immunofluorescence assay utilizing anti-SSEA-1 as the primary antibody.

B Undifferentiated EC cells persist, despite retinoic acid treatment. Fluorescence of a small cluster of EC-like cells in a culture treated 48 hr with retinoic acid. Cells with a differentiated morphology (seen faintly in the background) did not bind anti-SSEA-1.

The assays were performed 48 hr after plating small cell clumps from suspension cultures.



A



B

Table 13a IN VIVO: Embryo recovery data following incorporation of P19 cells

	untreated P19-morula aggregates	24 hr RA+ P19-morula aggregates	48 hr RA+ P19 injected blastocysts
embryos transferred	48	29	36
implants (% transferred)	33 (69%)	25 (86%)	32 (89%)
resorptions (% implants)	27 (82%)	16 (64%)	3 (9%)
embryos (% implants)	6 (18%)	9 (36%)	29 (91%)
% embryos normal	100	100	93
% embryos chimeric	0	0	0

Table 13b IN VITRO: Persistence of undifferentiated P19 cells in outgrowths

Outgrowth description	Persistence of undifferentiated EC cells in Day 2 outgrowths	
	detected by morphology	confirmed by immunofluorescence
untreated P19 cell clumps	8/8	6/6
24 hr RA+ P19 cell clumps	8/8	2/2
48 hr RA+ P19 cell clumps	18/19	2/2
		confirmation by autoradiography
untreated P19-morula aggregates	5/8	5/6
24 hr RA+ P19-morula aggregates	4/7	4/7
48 hr RA+ P19-morula aggregates	aggregation unsuccessful	
48 hr RA+ P19 injected blastocysts	not determined, all blastocysts transferred to recipients	

II AGGREGATIONS OF ICMs WITH MORULAE

1. In utero development of ICM-morula aggregates

These experiments were designed to test the effects of aggregating normal embryonic cell types, from two different developmental stages. In these experiments either a single inner cell mass (ICM) isolated from a 3.5 day blastocyst, or three such ICMs were aggregated with single 2.5 day morulae. Embryo recovery at 9.5 to 12.5 days gestation is given in Table 14.

Aggregate embryos containing ICM cells demonstrated a high rate of resorption not shared by the control morulae in these experiments. As the number of ICM cells in the aggregate was increased, the resorption rate also increased.

The ICM-morula aggregates which escaped early resorption and were recovered around midgestation (9.5 to 12.5 days) are described in Table 15. Embryos developing from single ICM-morula aggregates were all judged normal. Some of these embryos were left to develop longer than usual and were recovered at 12.5 gestation. They were as well developed as control fetuses at this stage.

Considerable variation was noted in the extent of colonization of the embryos by the added ICM cells (Table15). Five of the 13 embryos were not chimeric, and in four of these the added ICM cells had not colonized the membrane fraction either. In #10, the added ICM cells had colonized the membranes only. The other eight embryos were chimeric, with contributions ranging from about 40% to 100%. In each case where the added ICM cells had colonized the embryo fraction, they had also colonized the membranes, with contributions also ranging from 40% to as high as 100%. Small amounts of BB isozyme were detected in several of the ectoplacental cone fractions.

Table 14 Embryo recovery data following transfer of ICM-morula aggregates

	control morulae	ICM-morula aggregates	3ICM-morula aggregates
embryos transferred	65	48	36
implants (% transferred)	43 (66%)	34 (71%)	23 (64%)
resorptions (% implants)	7 (16%)	21 (62%)	20 (87%)
embryos (% implants)	36 (84%)	13 (38%)	3 (13%)

overall embryo recovery:

embryos recovered/embryos transferred

controls: 55%

ICM-morula aggregates: 27%

3ICM-morula aggregates: 8%

TABLE 15

Aggregations of ICMs with a morula

Aggregations of 1 ICM with a morula

Embryo and description	GPI Analysis		
	Embryo proper	Extraembryonic membranes	EPC
1. Normal			
2. Normal			
3. Normal			
4. Normal			
5. Normal			
6. Normal			
7. Normal			
8. Normal			
9. Normal			
10. Normal			
11. Normal			
12. Normal			
13. Normal			

Aggregations of 3 ICMs with a morula

1. Normal			
2. Disorganized, unfused allantois			
3. Normal			

EPC: ectoplacental cone fraction of the conceptus

The black portion of each diagram represents contribution by the added ICM cells.

Two of the three embryos recovered following aggregation of three ICMs with a morula were developing normally, but one embryo was clearly abnormal at 9.5 days development. This was a small embryo, with recognizable head fold and heart regions. The rest of the body appeared disorganized, with no recognizable somites, and an unfused allantois. The two normal embryos were very chimeric, as were their membranes. The abnormal embryo was difficult to dissect due to its small size, but was a chimera.

2. Substitution of egg culture media for ≪ tissue culture medium

2a. Development of early embryos in various culture media

The ICM-morula aggregates just described were cultured overnight in medium prior to transfer. In order to determine whether the high early rsorption rate seen was due to culture conditions, various egg (embryo) culture media were substituted. These defined media were devised to promote the growth of single and 2-cell embryos in vitro. Two egg culture media, one devised by Whitten (Biggers, et al., 1971) and the other by Brinster (Cross and Brinster, 1973) were selected. The compositions of these media and details of their preparation are given in Appendix 1.

In preliminary tests, the abilities of these media to support in vitro development of control embryos were compared. In the first test, embryos were collected at the 8-cell stage and cultured in ≪, Whitten's and Brinster's media. As shown in Table 16, blastocyst formation was somewhat delayed in both egg culture media.

In a second test, embryos were collected at the 2-cell stage, and cultured in both Brinster's and Whitten's media. The outcome is given in Table 17.

Table 16 Timing of blastocyst formation when 8-cell embryos were cultured in various media

Day 1	medium	Brinster's	Whitten's
Blastocysts	7	0	0
Cavititation beginning	4	5	5
Late morulae	7	4	1 + 2 dying embryos
Day 2			
Blastocysts	18	9	6
Cavitation beginning	0	0	0
Late morulae	0	0	0 + 2 dead embryos

Table 17 Timing of blastocyst formation when 2-cell embryos were cultured in various media

	2-cell	4-cell	6-cell	8-cell	compacted morulae	blastocysts
Day 1						
Brinster's	0	6	1	0	0	0
Whitten's	0	1	0	5	1	0
Day 2						
Brinster's	0	2	0	0	5	0
Whitten's	0	0	0	0	6	1
Day 3						
Brinster's	0	1	1	0	3	2
Whitten's	0	0	0	0	0	7

Development of these embryos from the 2-cell stage proceeded somewhat more rapidly in Whitten's medium, and all the embryos underwent successful blastocyst formation in this medium. These blastocysts appeared healthy and were subsequently transferred to a tissue culture surface where they formed normal blastocyst outgrowths.

2b. Subsequent *in utero* development of triple ICM-morula aggregates cultured in Whitten's medium during the preliminary in vitro period

Following the observation that early embryos developed best *in vitro* when cultured in Whitten's medium as opposed to Brinster's, this medium was chosen to test the effect of varying the *in vitro* conditions on subsequent development of ICM-morula aggregates. Control embryos and triple ICM-morula aggregates were transferred to recipients following 24 hr *in vitro* culture in this medium. The outcome of these experiments is given in Table 18. Embryo recovery at 10.5 days gestation following culture of control morulae in Whitten's medium was comparable to that seen following culture in ≺ medium. When 3ICM-morula aggregates were cultured in Whitten's medium prior to transfer, embryo recovery was still very low, but four embryos were recovered. All four appeared to be developing normally. One of these was as large and as well developed as the controls, and this embryo and its membranes were nearly completely derived from the added ICM cells. The other three embryos were very small at 10.5 days development, and the spots on the GPI analysis gels were faint, but these embryos were chimeras also.

Although no obvious difference was seen in embryo recovery following culture of the aggregates in Whitten's medium rather than ≺ medium, the low embryo recovery was due in this case primarily to a failure of these aggregates to implant in the uterus following culture in Whitten's medium. This difficulty

Table 18

A COMPARISON OF EMBRYO RECOVERY FOLLOWING CULTURE IN α MEDIUM VS WHITTEN'S MEDIUM

Description of Embryos	Embryos Transferred	Implants (% transferred)	Resorptions (% implants)	Embryos (% transferred)
Control morulae cultured in α medium	128	97 (76%)	17 (17.5%)	80 (62.5%)
3ICM-morula aggregates cultured in α medium	36	23 (64%)	20 (87%)	3 (8%)
Control morulae cultured in Whitten's medium (2 experiments: 5 recipients)	36	23 (64%)	3 (13%)	20 (55.5%)
3ICM-morula aggregates cultured in Whitten's medium	23	7 (30%)	3 (43%)	4 (17%)

was not shared by the controls cultured in this medium nor by aggregates cultured in α medium.

III Comparison of ICM-morula aggregate development to EC-morula aggregate development

1. *In vitro* development of aggregates

1a. Status of embryos following overnight *in vitro* culture

Aggregates were always cultured overnight *in vitro* prior to transfer to allow for the formation of a well integrated aggregate morula at a stage suitable for transfer to the recipient uterus. The status of aggregate embryos was assessed prior to transfer, following the overnight *in vitro* culture period. The status of ICM-morula aggregates is compared to controls and P10-morula aggregates in Table 19.

The histogram in Figure 8 compares the percentages of embryos in which blastocoele formation was at least detectable following the overnight culture period for the different types of aggregate embryos. 73% of control morulae, collected at the 8-cell stage the day previous and cultured overnight, had at least reached the late morula stage in which cavitation was detectable, and more than half of these were already blastocysts. The onset of cavitation was delayed in aggregates to which ICM or P10 cells had been added, with the addition of a single ICM having the least effect.

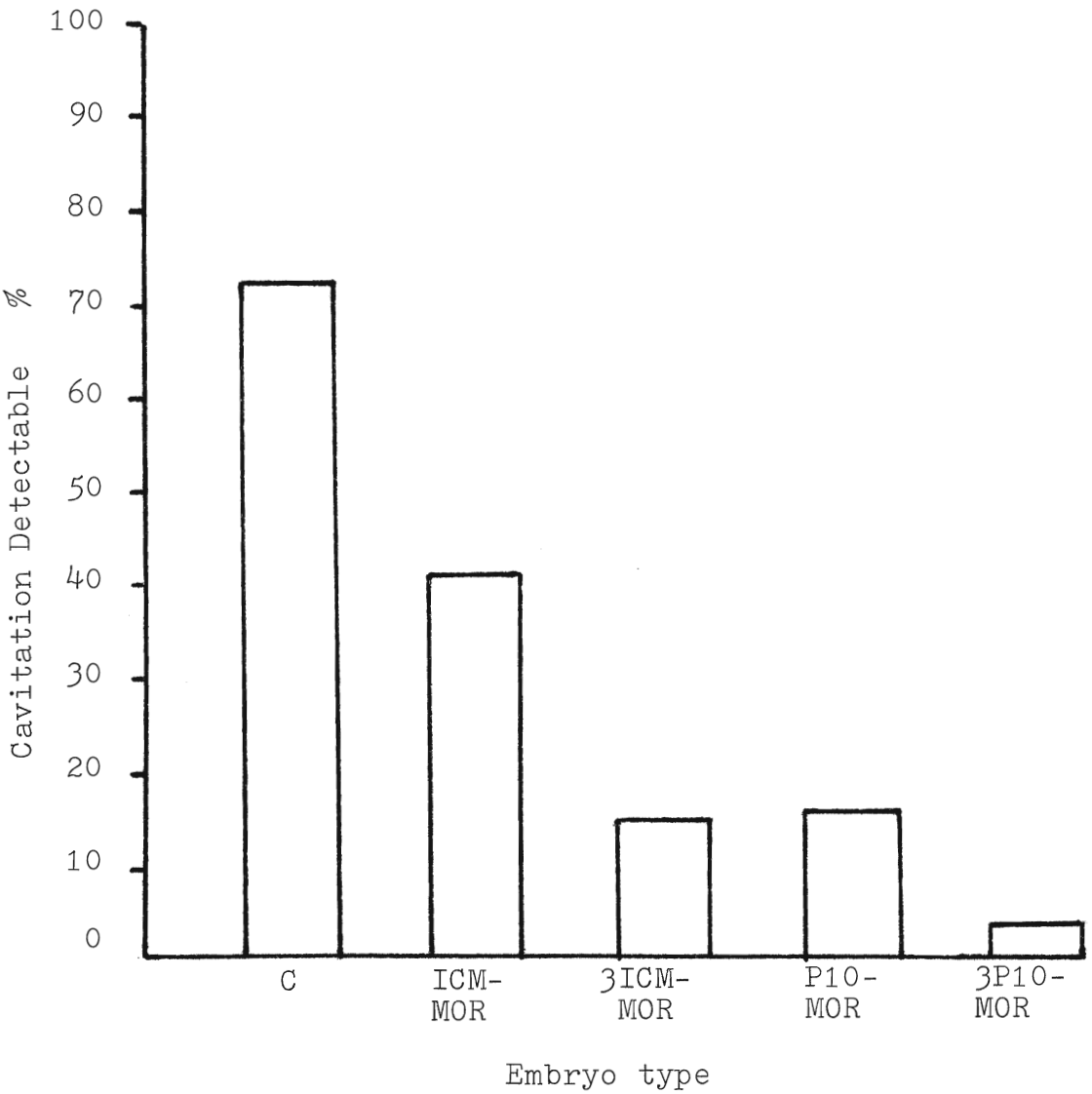
Where further development was to be assessed *in utero*, the embryos were transferred at this stage. In other experiments, embryos were allowed to proceed to the blastocyst stage *in vitro* by culturing a further 24 hr.

Table 19 Status of embryos following overnight *in vitro* culture

	Late morulae	Cavitation detectable	Blastocysts
Controls	27% (93/341)	28% (96/341)	45% (152/341)
ICM-morula aggregates	58% (63/108)	9% (10/108)	32% (35/108)
3ICM-morula aggregates	85.5% (71/83)	11% (9/83)	3.5% (3/83)
P10-morula aggregates	85% (77/91)	12% (11/91)	3% (3/91)
3P10-morula aggregates	97% (68/70)	3% (2/70)	0

FIGURE 8

STATUS OF EMBRYOS FOLLOWING OVERNIGHT IN VITRO CULTURE



C: control morulae ___-MOR: ICM or P10-morula aggregate

1b. Blastocyst formation *in vitro*

In 23 cases where triple ICM-morula aggregates were cultured 48 hr *in vitro* only one of the blastocysts which developed was considered to be morphologically normal when compared to control blastocysts. The unusual blastocysts exhibited a disproportionately large ICM (Plate 9). In other cases, the embryos barely resembled blastocysts and little evidence of trophectoderm formation was noted. However, when these unusual blastocysts were outgrown on tissue culture surfaces, giant cell differentiation was always evident.

In 17 cases where single ICM-morula aggregates were cultured 48 hr *in vitro*, seven blastocysts were judged to be morphologically normal, while the remainder had disproportionately large ICMs or unusual forms. Therefore, while the deviations from normal form were not as severe in this group, the majority of blastocysts were considered to be unusual.

P10-morula aggregates develop into large but normal blastocysts *in vitro*. In 23 cases where single P10-morula aggregates and 21 cases where triple P10-morula aggregates were cultured to the blastocyst stage *in vitro*, only two blastocysts were considered to be morphologically abnormal.

As already described, P19-morula aggregates generally do not form well integrated aggregate morulae in the overnight culture period. However, with further *in vitro* culture, these aggregates also develop into morphologically normal blastocysts (Plate 9). It is possible that some P19 cells may remain scattered on the outer surface of the trophectoderm.

Blastocyst formation by triple ICM-morula aggregates was also tested in the egg culture media. The blastocysts which developed from six such aggregates in Brinster's medium were unusual and comparable to those which formed in medium. When 3ICM-morula aggregates were cultured in Whitten's medium, the blastocysts which developed generally had a more normal appearance. Eleven

morphologically normal and only two unusual blastocysts developed from 13 aggregates cultured in Whitten's medium. The *in vitro* blastocyst formation results are summarized in Table 20.

1c. Blastocyst formation *in utero*

It must be stressed that when blastocyst formation was to be assessed *in vitro*, the embryos were cultured a total of 48 hours, whereas in experiments where embryos were transferred to recipients, the *in vitro* culture period was 24 hr only. As shown already in the histogram, most aggregate embryos are transferred at the late morula stage, and blastocyst formation takes place in the recipient uterus. In order to compare the blastocysts which develop from transferred 3ICM-morula aggregates in the uterus to those which develop *in vitro*, recipient uteri were flushed the day following embryo transfer. In two such experiments seven embryos were recovered following transfer of ten 3ICM-morula aggregates, and these all appeared to be normal late blastocysts which were about to implant in the uterus (Plate 9). That is, these blastocysts did not resemble the morphologically unusual blastocysts which developed when 3ICM-morula aggregates were cultured to the blastocyst stage *in vitro*. (Since EC-morula aggregates formed normal blastocysts *in vitro*, similar early recovery experiments were not done with these aggregates.)

Plate 9 Blastocyst formation from aggregates

A A blastocyst which developed in vitro in ∝ medium from a control morula.

B A morphologically unusual blastocyst which developed in vitro in ∝ medium from a triple ICM-morula aggregate.

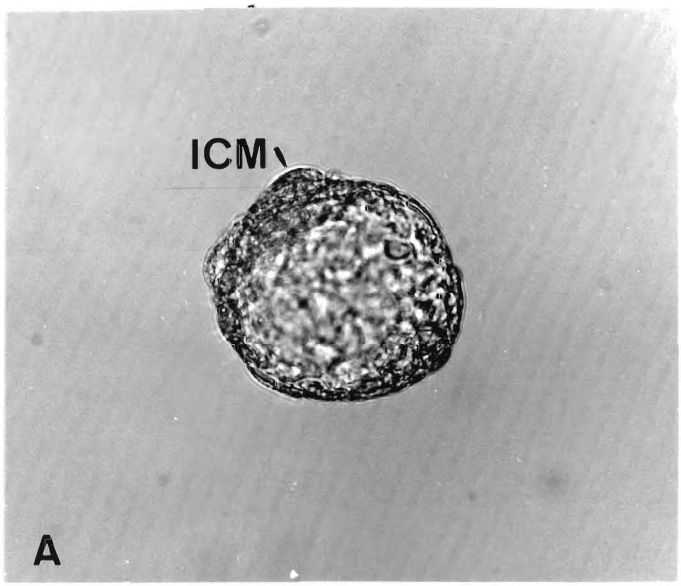
C A morphologically normal blastocyst which developed in vitro in Whitten's medium from a triple ICM-morula aggregate.

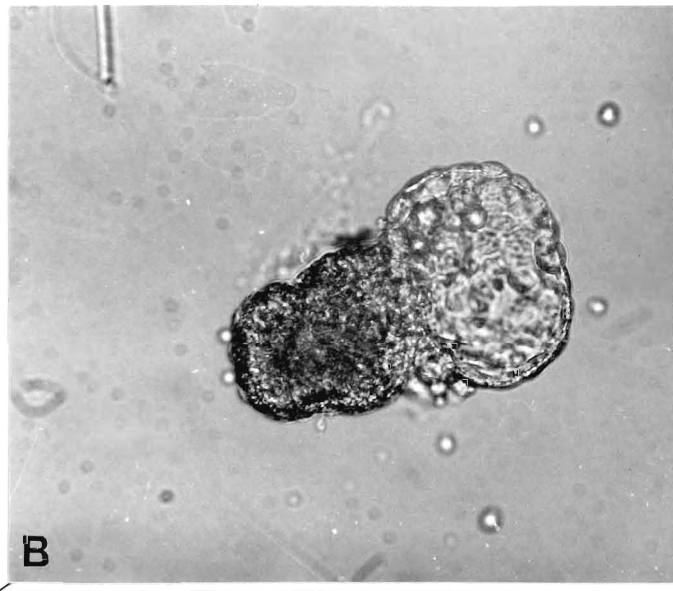
D Morphologically normal, late blastocysts flushed from the recipient uterus 24 hr after transfer of 3ICM-morula aggregates cultured in ∝ medium for only 24 hr prior to transfer.

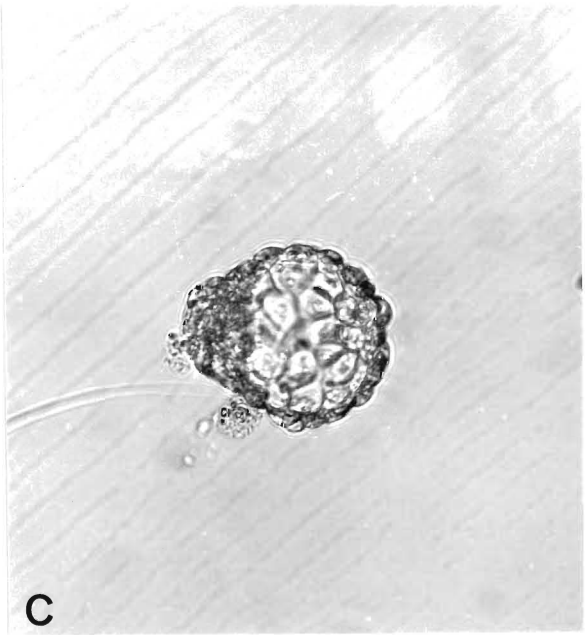
E A morphologically normal blastocyst which developed in vitro in ∝ medium from a 3P10-morula aggregate.

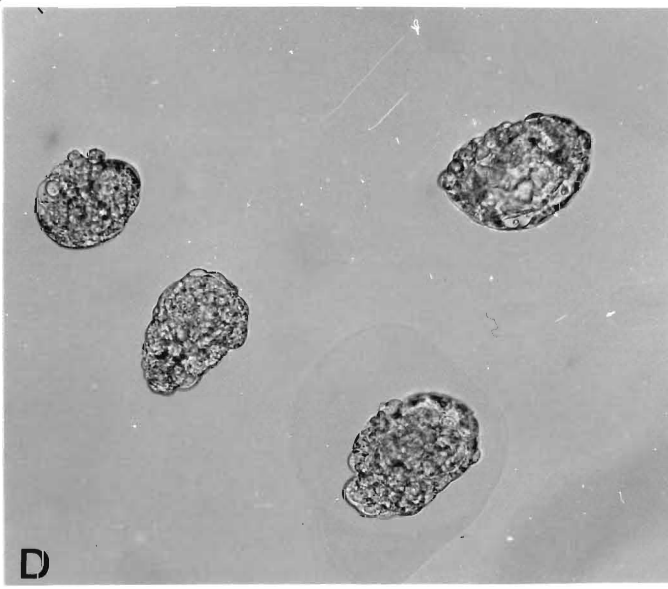
F Blastocysts which developed in vitro in ∝ medium from P19-morula aggregates. P19 cells may be scattered on the outer surface of the trophectoderm.

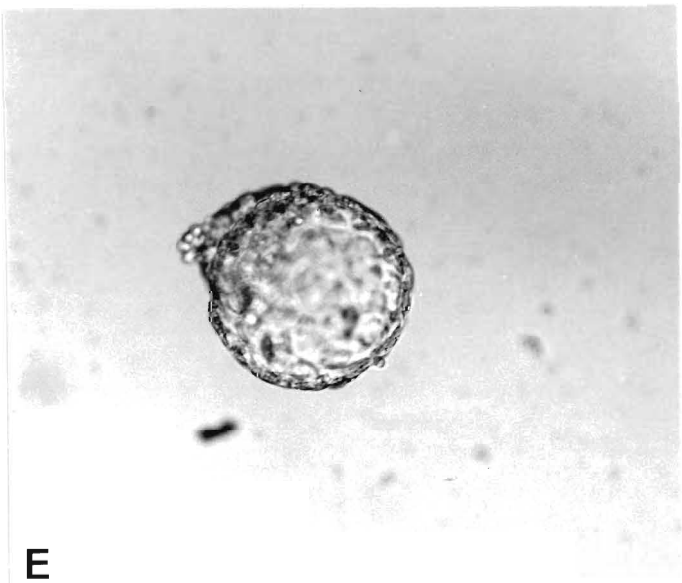
The blastocysts which developed in vitro were photographed following 48 hr cutlure beyond the 2.5 day morula stage.

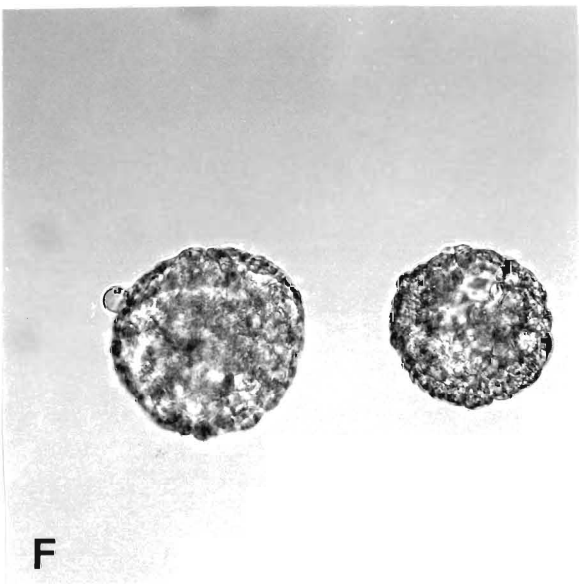












100 μm

Table 20 Blastocyst formation *in vitro*

Aggregate type	Embryos cultured to blastocyst stage *in vitro*	Morphologically unusual blastocysts
ICM-morula (inα)	17	10
3ICM-morula (inα)	23	22
3ICM-morula (Whitten's)	13	2
P10-morula	23	1
3P10-morula	21	1
P19-morula	9	0

2. Histological examinations of early post implantation development of EC-morula and 3ICM-morula aggregates

2a. 6.5 day histology

Ten implantation sites from two recipients of 3ICM-morula aggregates were examined at 6.5 days development. Eight of these sites contained debris only in the uterine crypt, while two embryos (one from each recipient) were recovered. These embryos were small and less well developed than controls at 6.5 days, but were apparently normal (Plate 10).

The development of P10-morula aggregates and P19-morula aggregates was examined at earlier stages for comparison with the ICM-morula histology. Eight 3P10-morula aggregate implantation sites were recovered following transfer of 15 aggregates to three recipients. In contrast to the ICM-morula aggregates, only two resorption sites were found at 6.5 days. An embryo had not developed in one implantation site, while the remaining five contained embryos. Four of these were small and gross abnormalities were not yet apparent. In one, a clearly abnormal embryo was present (Plate 11). In this embryo, the folding of the two primary germ layers is abnormal, such that two cavities appear to be forming, and the extraembryonic ectoderm appears somewhat deficient.

Five P19-morula implantation sites from one recipient were examined at 6.5 days. One embryo was found, which appeared normal. Two other sites were resorbing, while two more did not contain a recognizable embryo. One of these crypts is shown in Plate 11. A few giant cells can be found, as well as small round cells in the crypt which may be P19 cells.

2b. 5.5 day histology

Because both the ICM-morula and P19-morula aggregates were resorbing in large numbers before day 6, some of these embryos were examined at 5.5 days

development also.

Nine 3ICM-morula aggregates were transferred to two recipients in order to assess development at 5.5 days. Seven implantation sites were recovered. A variety of abnormalities were seen, ranging from various degrees of retardation in four small but apparently properly organized embryos, to poorly organized embryos, to one case in which only a few parietal endoderm cells could be identified within the trophoblast (Plate 11).

Eight P19-morula implantation sites were recovered at 5.5 days following transfer of ten aggregates to two recipients. These embryos generally did not develop even to the early egg cylinder stage. In six, a recognizable embryos could not be found, while two implantation sites contained small embryos.

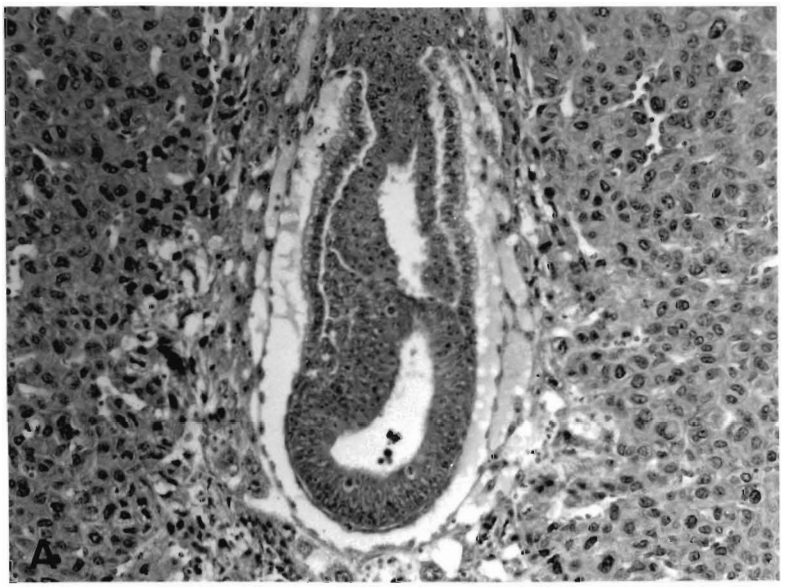
The histological findings are summarized in Table 21.

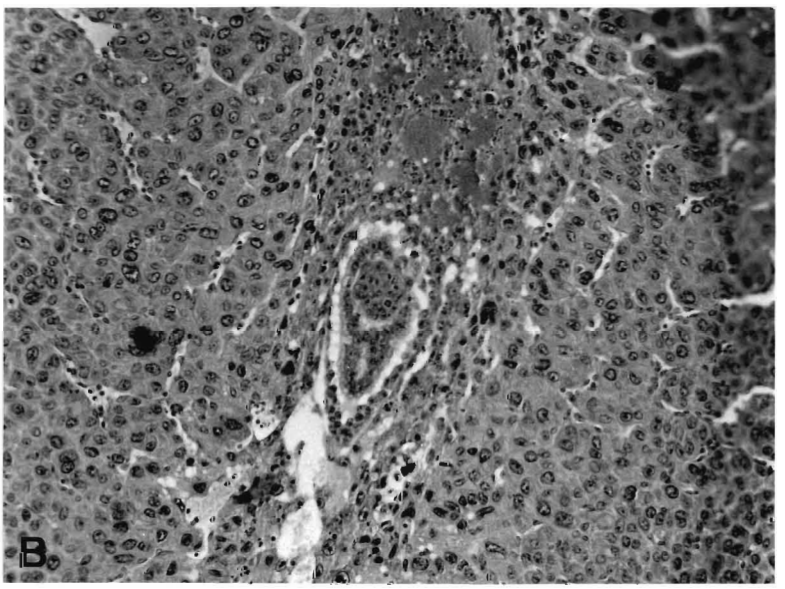
Plate 10 6.5 day histology of 3ICM-morula aggregates.

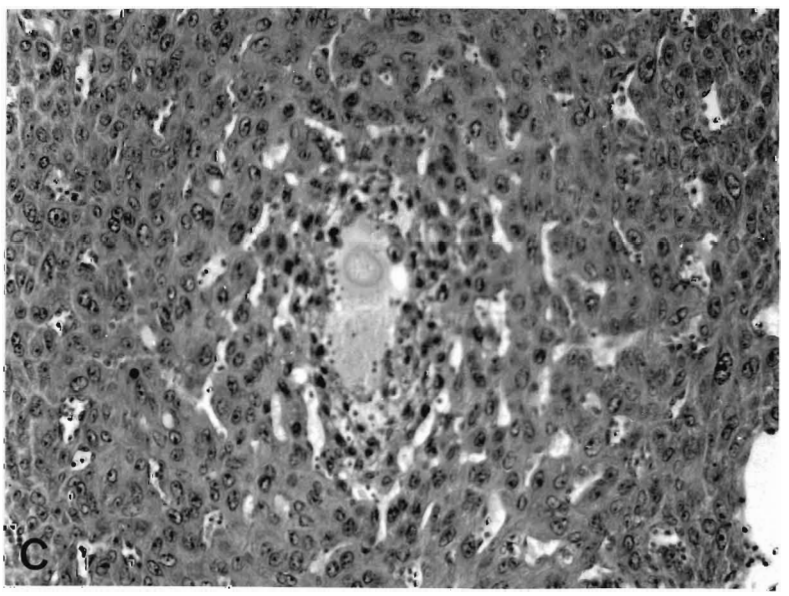
A A 6.5 day egg cylinder stage embryo developing from a transferred control morula. The embryo is at a stage of development comparable to the embryo diagrammed in Figure 2.

B An embryo developing from a 3ICM-morula aggregate at 6.5 days. While the form is overtly normal, the embryo is considerably retarded. 2 such embryos were found in 10 implantation sites.

C The remaining 8 implantation sites contained debris, indicating that the majority of 3ICM-morula aggregate embryos had died by 6.5 days.







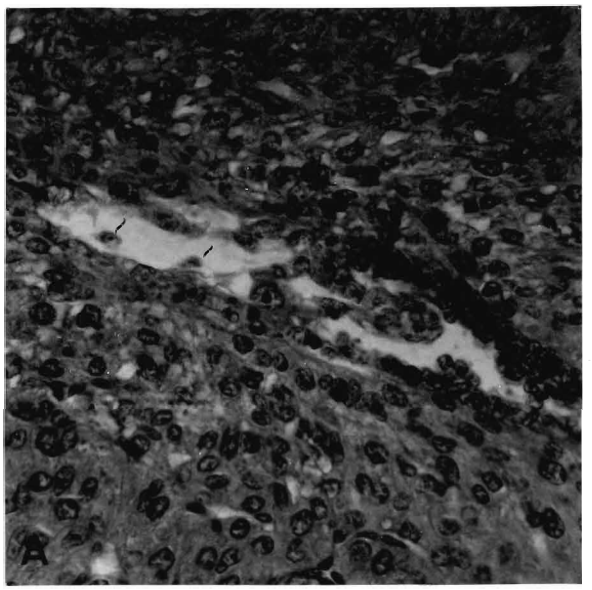
100 μm

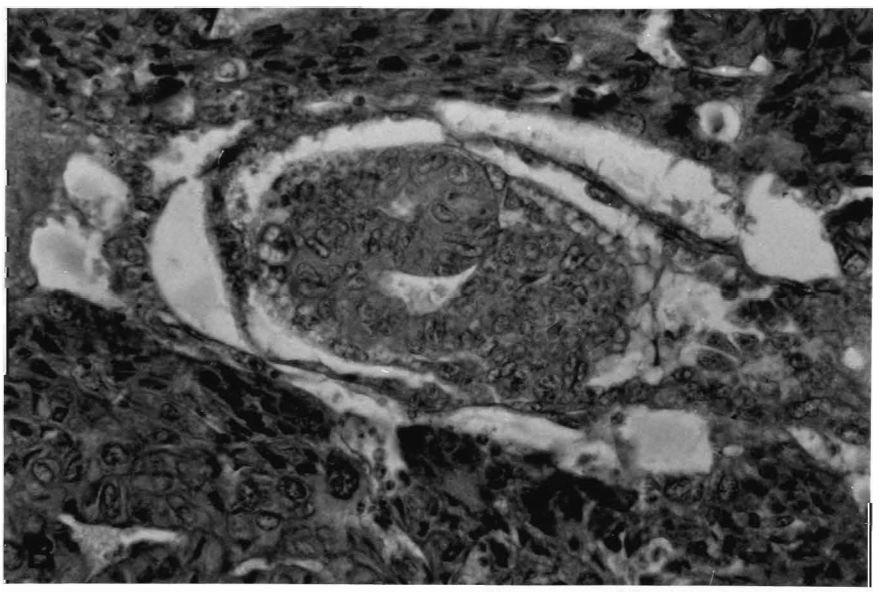
Plate 11 Examples of early abnormal development of ICM and EC containing aggregates.

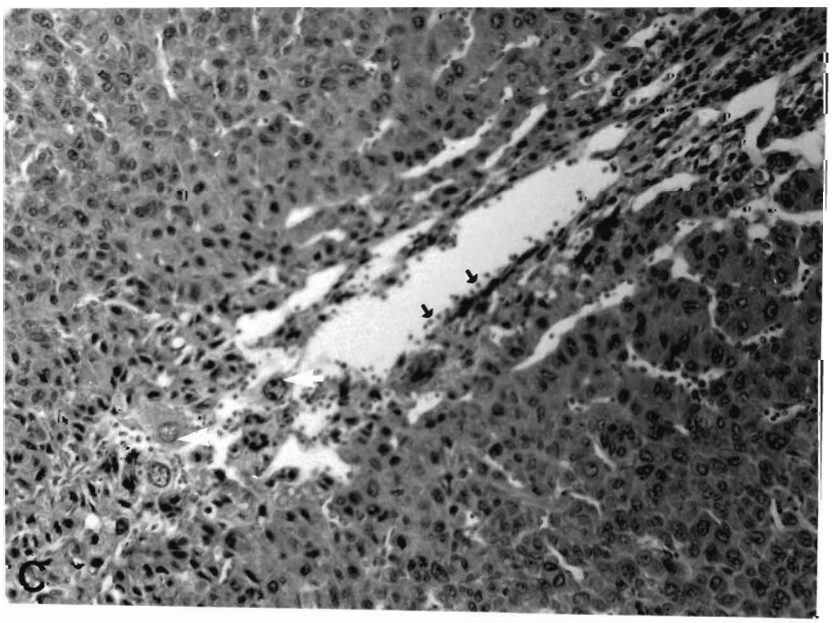
A A 5.5 day implantation site in which a 3ICM-morula aggregate had implanted. A properly organized egg cylinder has not developed. A few parietal endoderm cells can be identified (arrows).

B A 6.5 day implantation site containing an embryo developing from a 3P10-morula aggregate. The folding of the embryonic tissue is unusual, such that 2 cavities are forming.

C A 6.5 day implantation site in which a P19-morula aggregate had implanted. An egg cylinder has not developed. A few scattered embryonic giant cells can be identifed (white arrows). The small round cells (black arrow) within the crypt may be P19 cells.







50 μm

Table 21 Summary: Histological examinations

6.5 days	implant sites examined	normal embryos	abnormal development	resorptions
3ICM-morula aggregates	10	2 (small)	0	8
3P10-morula aggregates	8	4 (small)	2	2
P19-morula aggregates	5	1	2	2
5.5 days				
3ICM-morula aggregates	7	4 (small)	3	0
3P10-morula aggregates	-			
P19-morula aggregates	8	2 (small)	6	0

3. Comparison of embryo recovery following transfer of ICM-morula aggregates and P10-morula aggregates

Embryo recovery at midgestation of ICM-morula aggregates is compared to P10-morula aggregate recovery in Table 22.

Recovery of embryos at midgestation was much higher for P10-morula aggregates compared to recovery of embryos from ICM-morula aggregates. Although these P10 chimeric embryos were abnormal, they survived until at least midgestation, and recovery of embryos from P10-morula aggregates was not decreased as the number of P10 cells in the aggregate was increased.

These data have been subjected to statistical analysis by a χ^2 test (see Appendix 2). The differences between resorption rates of control morulae and P10 containing aggregate embryos are probably not significant ($p=<0.50$ and >0.20). The differences between resorption rates of control morulae and ICM containing aggregates are highly significant however ($p=<<0.001$).

TABLE 22

EMBRYO RECOVERY DATA

Description of embryo	Embryos transferred	Implants (% transferred)	Resorptions (% implants)	Embryos (% implants)
Control morulae	128	97 (76%)	17 (17.5%)	80 (82.5%)
Aggregate: P10 clump + a morula	41	29 (71%)	7 (24%)	22 (76%)
Aggregate: 3 P10 clumps + a morula	31	23 (74%)	6 (26%)	17 (74%)
Aggregate: single ICM + a morula	48	34 (71%)	21 (62%)	13 (38%)
Aggregate: 3 ICMs + a morula	36	23 (64%)	20 (87%)	3 (13%)

DISCUSSION

I The behavior of karyotypically normal EC cells in EC-embryo aggregation chimeras

Until this study, aggregations of fully euploid EC cells with morulae had not been made. McBurney's P10 and P19 EC cell lines seemed to be very promising candidates for a test of the ability of karyotypically normal EC cell lines to form aggregation chimeras. Their potential to colonize the embryo following blastocyst injection has been fully explored (Rossant and McBurney, 1982 and 1983) and P10 cells demonstrated considerable success in reverting to a normal embryonic phenotype in the blastocyst environment. It was predicted that following aggregation of P10 cells with 8-cell embryos, chimeras might be generated at an even higher frequency, and the P10 cells might predominate in at least some of these chimeras such that the in vivo developmental potential of this EC cell line could be more completely assessed. These two predictions concerning colonization of the embryo by EC cells following aggregation with morulae were borne out by the behavior of the P10 cells in this study. P10 cells colonized the aggregate embryos very successfully such that all the embryos recovered at midgestation were chimeric. Therefore, just as P10 has been the most successful EC line to colonize the embryo following blastocyst injection, it has now demonstrated the greatest capacity for producing chimeras following aggregation, exceeding the 79% chimeric embryo recovery rate (at midgestation) reported by Fujii and Martin following aggregation of PSA-1/NG2 cells with morulae. Secondly, each chimera recovered at midgestation exhibited a very high P10 contribution. Several embryos were totally, or almost totally P10 derived. However, such high levels of P10 contribution were incompatible with normal development. The only normal embryo recovered (#4 in Table 3) was

the only chimera with a P10 contribution of less than 50%. In the previous blastocyst injection experiments the live born chimeras never demonstrated a predominant P10 derived cell population, and the more highly chimeric embryos were obviously developing abnormally at midgestation. The results from these aggregation experiments lend considerably more support to the hypothesis that a majority population of normal embryonic cells may be required in an EC-embryo chimera to provide a proper developmental framework.

Fairly high numbers of P10 cells (15 to 20) were aggregated with each 8-cell embryo in the first series of experiments. Because it had been demonstrated already that the embryo could sometimes regulate this cell load following blastocyst injection, it was not unreasonable to begin by aggregating a similar number of P10 cells with morulae. It has been suggested that the regulatory capacity of the embryo decreases as the number of EC cells added is increased (Martin, 1980; Pierce, et al., 1979; Illmensee and Stevens, 1979; Dewey, et al., 1977). In order to test for a dose-response relationship between the number of P10 cells aggregated and the degree of abnormality, the effect of increasing the number of P10 cells present in the aggregate at the outset was first examined by aggregating three ICM-sized clumps of P10 cells with each embryo. Despite the imposition of this much larger EC load on the embryos, development was no worse. Survival time of these chimeras did not decrease, nor did the degree of overt abnormality increase. Their development was retarded relative to the first set of P10 chimeras, such that very small embryos were recovered at 9.5 days gestation, but they achieved a comparable stage of development by 11.5 days. The abnormal chimeras probably arrest around this stage of development and die shortly thereafter, judging by total resorption in two attempts to recover chimeras beyond 12.5 days.

Interestingly, these very small 9.5 day embryos were less chimeric than the

embryos developing from the single P10-morula aggregates. By 11.5 days, however, these embryos were heavily chimeric. Initially P10 proliferation in the embryos may be relatively slow, such that P10 cells take over gradually in the developing chimeras. This hypothesis could be tested in a limited fashion by evaluating P10 contribution in developing chimeric embryos from even earlier stages, but it is unlikely that sufficient embryonic tissue would be present much earlier than 9 days for GPI testing without pooling embryos. The development of an _in situ_ marker to distinguish EC derived cells in chimeric tissue would be more desirable.

The effect of decreasing the P10 component of the aggregate at the outset was next tested. It has been suggested by the critics of high EC proportions that an embryo can be expected to regulate about three to five EC cells. Difficulties in aggregating such small numbers of EC cells were anticipated, primarily since the selection of undifferentiated P10 cells from mixed cultures of feeders, EC cells, and differentiated P10 cells depended on the morphology of ICM-like clumps of EC cells. Taking note that aggregating three to five EC cells with an 8-cell embryo would establish an EC to embryo cell ratio of 1:2, the usual clump of P10 cells, containing about 15 to 20 cells, was aggregated with two 8-cell embryos. Such an aggregate contained EC cells and embryonic cells in about equal proportions at the outset. A trend towards improved development was not noted. The embryos recovered at midgestation were just as chimeric, and just as abnormal as those in previous aggregations. Stewart (unpublished) recently aggregated only two or three P10 cells with two morulae, and while a few live born P10 aggregation chimeras were recovered (with P10 contributions less than 50%) losses were still very high during gestation. The results from this study predict that the embryos lost were abnormal chimeras with predominant P10 populations. Apparently, P10 cells very successfully

colonize the embryo following aggregation with morulae such that varying the initial P10 proportion in the aggregate (from 6:1 to 1:1) does little to reduce the eventual colonization of the embryo and is of little importance, then, to the outcome at midgestation.

The P10 cells did not colonize the extraembryonic membrane fraction to the same extent as the embryo proper. P10 contribution to this fraction never exceeded 50%, even in the embryos developing from triple P10-morula aggregates. Stewart (1982) also dissected conceptuses into their component fractions in his aggregation study. (The yolk sac has two components, extraembryonic endoderm and extraembryonic mesoderm, and these can be separated enzymatically by 13 days gestation.) Stewart found that EC cells often preferentially colonized the yolk sac mesoderm, and suggested that cultured EC cells require time to adjust to the embryonic environment and may not have differentiated endoderm. Since P10 aggregation chimeras do not persist to 13 days, it was not possible to determine if these cells also preferentially colonize the yolk sac mesoderm following aggregation. However, Rossant has separated the yolk sac layers in the longer surviving blastocyst injection chimeras, and has also noted preferential colonization of the yolk sac mesoderm by P10 cells. She points put that P10 cells will form some endoderm in aggregates, and readily differentiate endoderm in response to retinoic acid. Cell cycle considerations may be important, then, during the integration of P10 cells into development. P10 cells in culture clearly have a slower growth rate than other EC cell lines, such as P19 or PSA-1 (Rosenstraus, et al., 1984) which exhibit doubling times of about 12 hr. Papaioannou, et al. (1978) point out that by 6.5 days development a proliferative burst occurs in the embryo (confirmed by Lewis and Rossant, 1982) such that the average cell cycle time is 5 hr and cell cycle times as little as 2.5 hr are seen in certain regions. Therefore, they suggest that an

injected EC cell faces a considerable challenge in that it must shorten its cell cycle time fairly rapidly if it is to contribute significantly to a developing embryo. Aggregation of P10 cells with earlier 2.5 day embryos should have an advantage in this regard over injection into 3.5 day blastocycts. However, the cells may still not participate to an extensive degree in early developmental events such as primitive endoderm formation, which occurs shortly before implantation. By the time of mesoderm formation (about 3 days after implantation) they may have adjusted their cell cycles, allowing them to form mesoderm and eventually predominate in the embryos. The embryo probably becomes progressively more disorganised with increasing P10 participation, and while it survives the egg cylinder stage, it may not undergo proper organogenesis due to limitations in the ability of the P10 cells to direct normal development.

P10 cells also did not contribute to the trophectoderm derived ectoplacental cone (epc) fraction of any conceptus beyond a trace in four samples. These trace contributions are most likely due to contamination by adherent membranes. Stewart saw no colonization of trophoblast tissue by his EC cell lines following aggregation, and Rossant saw trace contributions only to a few P10 injection chimera epc fractions. A similar explanation for the failure to colonize this fraction could be made. Trophectoderm formation occurs very shortly after aggregation, and has already occurred in blastocyst injection experiments, such that EC cells might not be expected to participate. But in light of the embryonic ectoderm origin of EC cells, it seems more likely that EC cells share the developmental restriction acquired by ICM cells; that is, they are no longer totipotent, but are determined cells which have lost the capacity to differentiate trophectoderm.

P19 is another diploid EC cell line, and the behavior of these cells in aggregation chimeras was compared to that of P10. Although the embryos were abnormal, P10 chimeras survived until midgestation. In striking contrast, P19-morula aggregate embryos resorbed in very high numbers prior to midgestation. The only embryos recovered were normal and non-chimeric. Previously, in blastocyst injection experiments, P19 cells also performed poorly compared to P10, but abnormal chimeric embryos were recovered at midgestation (Rossant and McBurney, 1982, 1983). Embryo loss was high after this time and the few live born chimeras had tumors. Rossant and Papaioannou (in press) have now compared the in vitro outgrowths of blastocysts injected with either P10 or P19 cells. EC cells grew out from most (62/72) blastocysts injected with P19 cells but rarely (7/81) from blastocysts injected with P10 cells. These results suggest that P19 cells might not be regulated as well by the blastocyst. Perhaps they do not respond as well to signals to differentiate and instead persist as undifferentiated EC cells within the embryos. Was persistence of undifferentiated EC cells responsible for the early failure of the great majority of the P19-morula aggregation chimeras?

It was difficult to prove that proliferating P19 EC cells were disrupting development very early following aggregation since no evidence for EC contribution to development was found. In a few cases, material recovered from resorption sites was run on gels, but very little debris was present and only AA isozyme was detected. This was probably maternal enzyme. Recovery of such small portions of debris was understandable following the histological examinations. They revealed that embryo development ceased very early and egg cylinders usually failed to develop. The few embryos which were recovered at 5.5 or 6.5 days were normal and presumably non-chimeric.

P19 cells, with a fairly short cell cycle time in vitro (about 12 hr) might

proliferate rapidly in the aggregates from the outset. There is increasing evidence that differentiation of EC cells is associated with a lengthening of the cell cycle, and that, specifically, a growth arrest must occur in G1 (Rosenstraus, et al., 1982; Scott, et al., 1982). P19 cells may be less capable than P10 cells of leaving the proliferative phase and participating in development in a chimera. We must remember that EC cells have been selected for the property of continued proliferation, and feeder independent, fast-growing P19 cells seem to have adapted well to in vitro proliferation as stem cells. These cells may have lost much of the capacity to respond to the signals in the embryo which normally induce differentiation of pluripotent cells (Papaioannou and Rossant, 1983). Proliferating P19 cells may therefore disrupt the development of the embryo very early, preventing even egg cylinder formation. The recovery of a few normal, non-chimeric embryos from P19-morula aggregates is curious. Perhaps these cases reflect the tendency of P19 cells to form less well integrated aggregates. P19 cells injected into trophoblast vesicles die and only debris remains within the trophoblast a few days after transfer (Rossant and Papaioannou, in press). Similarly, if P19 cells do not integrate with the ICM of the embryo, they may die in these cases also.

Fujii and Martin (1983) suggested that aggregation with morulae might represent a simple alternative to blastocyst injection for producing EC-embryo chimeras. Their aggregation chimeras also showed high EC contributions, and a high degree of abnormal development was seen in those chimeras, suggesting that high EC contributions were incompatible with normal development. But at the time of their study, very few euploid EC cell lines were available, and they, and Stewart (1982) also, had tested EC cells with known karyotypic abnormalities, the most serious of which was a lethal trisomy. The presence of this trisomy in a large proportion of the cells in the developing chimeric

embryos might have adversely affected their development.

P19 cells retain a stable male diploid karyotype even with prolonged culture, and the only alteration noted to the normal P10 female karyotype has been the loss of an X-chromosome from some of the cells. That is, P10 cells sometimes acquire an XO genotype, with 39 chromosomes rather than the normal murine complement of 40 (G. Paterno and M. McBurney, unpublished). At the completion of this study, chromosome preparations were made from the P10 cells currently in culture. This culture had been maintained about two months, and in keeping with McBurney's observations, it was found to contain a mixture of cells with 39 or 40 chromosomes. The analysis of G-banded chromosomes in one spread containing 39 chromosomes indicated that the cells were XO. X-chromosome instability in cultured cells appears to be a rather common phenomenon. Not only are many EC cell lines XO, but pluripotent cells cultured directly from the embryo have also been shown to acquire the XO genotype with continued culture (Robertson et al., 1983). XO mice are healthy females which retain some fertility, although oogenesis is impaired (Burgoyne and Baker, 1981). While acquisition of the XO genotype might mitigate against germ line contribution, as Rossant has suggested (in preparation), it would seem very unlikely that this aberration was responsible for the gross developmental abnormalities seen in the chimeric embryos recovered in this study.

Therefore, this study is the first in which the ability to form aggregation chimeras has been tested for EC cells without potentially deleterious chromosomal abnormalities. Furthermore, this study is the first in which the aggregation results could be compared to a complete set of results from blastocyst injections of the same EC cell lines. The results from the blastocyst injection studies suggested that a high EC contribution correlated with abnormal development. Aggregations of these same cell lines produced an

even higher rate of abnormal development, and in the case of P10 cells, it could be demonstrated that the abnormal embryos had very high EC contributions, strengthening the hypothesis considerably that high EC contribution is incompatible with normal development. It is possible, however, that at least some of the developmental difficulties encountered by EC-embryo aggregation chimeras might have been due to developmental asynchrony between the two cell types in the aggregates. This point will be considered in the next section.

II The behavior of ICM cells in aggregation chimeras

Were the abnormalities characteristic of the EC-embryo chimeras due solely to the presence of a majority population of EC cells of tumor origin? EC cells appear to most closely resemble the pluripotent embryonic ectoderm of the late blastocyst and early egg cylinder and are clearly different from the blastomeres of the cleavage stage embryo. Therefore, when EC-morula aggregates were constructed, a mismatching of embryonic cell types was created. What are the effects of mismatching temporally asynchronous embryonic cell types on subsequent development? In order to create a control situation to test this question, aggregates had to be constructed which contained only normal, non-tumor derived embryonic cells, from different developmental stages. ICMs, isolated by immunosurgery from 3.5 day blastocysts, were aggregated with 2.5 day morulae and these aggregates were tested for normal development in the parallel study to the EC-morula aggregations.

The comparison of embryo recovery data (Table 22) revealed striking differences in the early resorption rates between P10 containing aggregates and ICM containing aggregates. P10 containing aggregates, even those containing three ICM-sized P10 clumps, implanted in the uterus and survived until midgestation at the same rate as transferred control morulae. However, when a single ICM (containing 15 or 20 normal embryonic cells) was aggregated with a morula, 62% of the embryos died and were resorbed prior to midgestation, a statistically significant difference in resorption rate. Unlike the P10 experiments, in which no dose-response effect could be demonstrated by varying the numbers of P10 cells aggregated, the addition of three ICMs to a morula resulted in an even higher resorption rate (87%) such that embryo recovery at midgestation was very low.

There have been other reports of low embryo recovery in similar types of

experiments. Fujii and Martin (1983) aggregated single ICMs with morulae and recovered embryos at a low rate (21 embryos/74 transferred = 28%). However, embryo loss was due to a failure of the embryos to implant, not to subsequent resorption, and control morulae implanted at the same inexplicably low rate (28%) in that study. Many of the embryos recovered were heavily chimeric and normal, while a few were somewhat retarded relative to the controls. While they conclude that they may have some evidence for altered development of aggregates containing embryo cells of different stages, this conclusion was not completely justified from their data. The results from this present study more convincingly demonstrate problems in development of these aggregates, due to embryo recovery rates significantly lower than controls and the recovery of one clearly abnormal embryo.

Rossant has also noted low embryo recovery following aggregation of ICMs with morulae. In her first study, (1975) the implantation rates of these aggregates were consistently lower than the controls, and in two later studies (Rossant and Lis, 1979a and b) about half of the aggregate embryos resorbed early. Embryo recovery was considerably lower then, than that seen in her blastocyst injection experiments, but those data were not compared to recovery rates of transferred morula controls. This present study confirms this trend towards lower embryo recovery by making the comparison with controls, and by demonstrating a dose-response effect in the aggregations of three ICMs with a morula.

Thirteen embryos escaped the early resorption effect following aggregation of an ICM with a morula. In this series of embryos, two major deviations from the embryos recovered after aggregating EC cells were seen. Unlike embryos recovered following aggregation of P10 cells, these embryos were all developing

normally. Unlike the normal embryos recovered following the aggregation of P19 cells, many of these normal embryos were chimeric. In fact, the added ICM cells had totally directed the normal development of an embryo and its membranes in some cases.

Three embryos only were recovered following aggregations of three ICMs with a morula. Two of these were normal and highly chimeric, but the third embryo, containing only normal, non-tumor derived cells, was clearly abnormal at midgestation. It exhibited abnormalities such as the poorly organised body and unfused allantois characteristic of P10 chimeras, suggesting that embryonic cells are capable of the same incomplete or inappropriate response to developmental signals in certain circumstances.

Trace contributions of BB isozyme were seen in several ectoplacental cone fractions. While early ICM cells can colonize the trophectoderm in chimeras, no particular selection for early ICMs was made in these experiments, and these trace contributions are more likely to represent contamination by adherent membranes from the larger, more developed embryos recovered in these experiments. Correlating with this explanation is the observation that when the membrane fraction was not chimeric, no contribution to the epc was seen.

In answer to the question raised at the beginning of this section, it was apparent that there were problems associated with the development of aggregate embryos containing only normal embryonic cells taken from different stages of development. However, the problems were not the same as those seen in the development of EC-morula aggregation chimeras, and it seems most unlikely that the developmental difficulties experienced by EC-morula aggregation chimeras were due solely to developmental asynchrony.

Given that some normal ICM-morula aggregation chimeras could be recovered, why were so many of these embryos lost? Earlier stages of their development

were examined and compared to EC-morula aggregates at similar stages. It was noted that the addition of either ICM or EC cells to a morula delayed the onset of cavitation, but the effect was most marked in the P10 containing embryos. Eventually, these P10 containing embryos did develop into morphologically normal blastocysts *in vitro*. Interestingly, ICM containing embryos formed morphologically abnormal blastocysts in vitro. The appearance of these blastocysts suggested that the proliferation of the ICM cells was not regulated, such that blastocysts with disproportionately large ICMs, or blastocysts overgrown with ICM cells developed. While the formation of an improperly organized blastocyst could certainly account for the early failure of these embryos, these blastocysts had developed during a 48 hr *in vitro* culture period, while transferred embryos were maintained *in vitro* for 24 hr only and transferred usually as late morulae. When blastocysts which had developed from these late morulae were recovered from the uterus, the ICM cell proliferation appeared to have been regulated within the uterine environment, suggesting that the grossly abnormal blastocysts may have been *in vitro* artifacts. Indeed, when the *in vitro* culture conditions were changed by varying the culture medium, normal blastocysts developed *in vitro* in Whitten's egg culture medium. Abnormal blastocysts had developed in another defined egg culture medium, and when the compositions of Whitten's and Brinster's media are compared, the greatest differences appear to be in the Na concentration, and in the amount of bovine serum albumin added. Brinster's medium contains 0.5% BSA vs 0.3% BSA in Whitten's, and Brinster's medium would have a higher osmolarity due to both the higher Na and protein concentrations. Perhaps these are conditions which are more conducive to eventual excessive ICM cell proliferation *in vitro*.

Having seen the development of morphologically normal blastocysts from 3ICM-morula aggregates *in vitro* in Whitten's medium, this medium was substituted

for ∝ medium in the 24 hr in vitro culture period prior to transfer to determine if embryo recovery rates were improved. Previous to these experiments, Brinster's and Whitten's egg culture media had been tested for their ability to support in vitro embryo development. Both media allowed the development of blastocysts from embryos collected at the 8-cell stage, at a somewhat slower rate than that in ∝ medium. Whitten's medium appeared superior to Brinster's for the culture of embryos from the 2-cell stage. Following transfer of embryos cultured in Whitten's medium, control embryo recovery was comparable to that seen when the embryos were cultured in ∝ medium prior to transfer.

The recovery of four embryos following the transfer of 23 3ICM-morula aggregates cultured in Whitten's medium may represent an improvement over recovery of three embryos following transfer of 36 3ICM-morula aggregates cultured in ∝ medium. However, embryo loss was still very high, and another variable appears to have been introduced with the use of Whitten's culture medium. Embryo loss was due primarily to a failure of these aggregate embryos to implant in the uterus, a difficulty not shared by Whitten controls in this series of experiments, nor by aggregates cultured in ∝ medium. It is interesting that in one of Rossant's earlier studies of ICM-morula aggregation chimeras (1975), the implantation rates of the aggregates were consistently somewhat lower than the control rates. These aggregates were cultured in a similar egg culture medium (M16), but usually for 4 hr only prior to transfer. These current results confirm the earlier finding, and the trend towards lower implantation appears to have been magnified by the longer in vitro culture period.

Given that grossly abnormal blastocysts apparently do not develop from ICM-morula aggregates in the uterus, and that varying the in vitro culture conditions such that normal blastocysts develop in vitro does not "cure" these

aggregates with respect to in vivo embryo recovery, it seems unlikely that the formation of an improperly organised blastocyst was the overriding reason for early embryo failure. The histological examinations indicated that resorption of these ICM containing aggregate embryos occurred very early, before 6.5 days in the majority of cases. The histological examination of 5.5 day embryos did not reveal a recurring developmental defect, but established that a variety of deviations from normal development could be detected at this time. In several cases, retardation was the only obvious deviation, but in light of the very low embryo recovery at midgestation, it would seem likely that some of these apparently normal but retarded embryos would not survive. Although both P19 and ICM containing aggregates fail in large numbers before midgestation, a difference in early development of these aggregates was noted, in that very little, if any egg cylinder development was seen in P19 implantation sites.

To summarize, a comparison of ICM-morula and EC-morula aggregate embryo development revealed that abnormalities of development are not the same amongst the various aggregates examined.

1. The in vitro tests of development indicated that EC-morula aggregates form normal blastocysts but that ICM-morula aggregates sometimes formed abnormal blastocysts, depending on the culture medium employed.

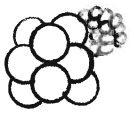
2. The early post-implantation development of P10 containing embryos was the most normal amongst the various aggregate embryos. Abnormalities in egg cylinder development were more obvious for ICM-containing aggregates, while P19-containing aggregates usually did not reach even the early egg cylinder stage.

3. By midgestation, the greatest differences amongst the embryos were seen. P10 containing embryos survived until midgestation, but were grossly abnormal. Most P19-morula aggregates did not survive until midgestation. The embryos recovered at midgestation were normal and non-chimeric. ICM containing embryos also demonstrated a high resorption rate prior to midgestation, but in contrast to EC-morula aggregates, it was possible to recover some normal chimeric embryos from ICM-morula aggregates.

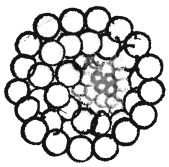
An hypothesis will be now be proposed to try to explain the low survival rate of ICM-morula aggregates and the differences in outcome following aggregation of P19 and P10 cells with morulae.

What happens to the ICM following aggregation? ICM cells, the inner cells of the blastocyst, acquire a set of surface properties distinct from outer cells (Ziomek and Johnson, 1980 and 1982) and these inner properties can be recognized by cleavage stage blastomeres (Surani and Handyside, 1983). These cells spread over the more adhesive ICM cells, and during formation of an aggregate, the ICM will eventually be engulfed by the blastomeres, and internalised (Kimber et al., 1982). Surani and Barton (1984) review these experiments, and point out that EC cells evoke the same responses. As Stewart demonstrated in his first aggregation experiments, EC cells are also internalised in EC-morula aggregates, and this has been confirmed recently by Lehtonen et al. (1984). (In these present studies, there was evidence that EC cells might differ in this regard, in that P10 cells appeared to form well integrated aggregate morulae and presumably were more readily internalised than P19 cells.) Surani and Barton suggest that the total number of cells inside an aggregate determines whether further cells will be recruited from the still labile pre-blastocyst outer cell

population (the host cell population). A model of an aggregate, and certain predictions can be developed, following a consideration of Barton and Surani's hypothesis.



aggregation of an ICM or EC clump with a morula



cross section through a hypothetical aggregate at the late morula stage

In the cross section through a hypothetical ICM or EC-morula aggregate at the late morula stage, three cell populations would be expected. The ICM or EC clump has been internalised and is surrounded by host blastomeres (the black cells). The ICM or EC clump consists of two cell populations, the outer cells (blue) and a small group of three to five inner cells (red) which, in the case of the ICM of a normal blastocyst, would give rise to the embryo, while the outer cells would give rise to the membranes. I would suggest that this model of an aggregate predicts that the added ICM or EC cells will give rise to the embryo and contribute substantially to its membranes even if the added ICM cell population has not expanded very much in the initial period after aggregation. The probability that an outer host blastomere would be recruited not only to the inner compartment but to the already established inner compartment of the ICM (that is, to the red area) would seem rather low. Yet, the pattern of colonization of the 13 embryos recovered at midgestation following aggregation of a single ICM with a morula does not fit these predictions. Only two embryos were recovered which were totally ICM-cell derived. Six other embryos were true chimeras, indicating that at least one host blastomere was recruited to the inner compartment of the blastocyst. And, in one conceptus, the embryo was

entirely host derived while the membranes were chimeric. In four other conceptuses, the added ICM cells did not participate in development at all. Even when three ICMs were aggregated with a morula, host blastomeres still participated in development. These results seem quite peculiar when this model is considered. In Rossant's three previous papers, embryos derived totally from the added ICM were seen, but more than half of the chimeras recovered had very substantial host blastomere contributions, and a rather large number of non-chimeric, host derived embryos were recovered in those experiments.

These observations on colonization of chimeras suggest that the ICM cells are not integrated into development to the degree that we would expect following aggregation such that host blastomeres are recruited for embryo development. The majority of the ICM containing aggregates die, apparently after reaching the early egg cylinder stage. Why do they not continue their development, giving rise to non-chimeric, normal embryos, if the added ICM cells are simply excluded from the developing embryo in most cases? The ICM cells have been exposed to the microenvironment in the 3.5 day blastocyst and have been "primed" to differentiate. Therefore, it is possible that the added ICM cells cannot reintegrate into the 2.5 day embryo. Instead, they may continue along their already determined pathway, seriously disturbing the cell-cell interactions in the morula, leading to early embryo failure in most cases. Sometimes though the proper interactions must be established in these aggregates such that development proceeds, giving rise to normal embryos which often are chimeric.

One possible incompatibility between ICM and morula cells may be in gap junction formation. Communication, and metabolic cooperation between cells in an embryo are normally established via gap junction formation beginning at compaction. Unless gap junctions are established between the internalised ICM

cells and the host blastomeres in the aggregates, these cells might not interact properly with each other. The possible role of gap junction formation between cells in a chimera as a factor influencing subsequent development has been considered recently by Papaioannou (Papaioannou, Waters and Rossant, submitted). She found that both P10 and P19 cells formed functional gap junctions with ICM cells and also with the blastomeres of morulae in co-cultures, and therefore a difference in gap junction formation (and communication) between these cells cannot explain the subsequent differences in development of these chimeras. Do gap junctions form between the cells in ICM-morula aggregation chimeras? A careful electron microscopic study of sectioned late aggregate morulae, such as the one described recently by Lehtonen et al. (1984) in which EC-morula aggregates were examined, may be required to examine the interactions between these cells.

Do EC cells behave differently than ICM cells in the aggregates during this initial early period? Unlike ICM cells, EC cells may not be committed to differentiation. They have been selected for the property of continued proliferation, and in the aggregate embryos, they may continue proliferation as stem cells. In the case of P19 cells, this proliferation of stem cells is apparently deleterious very early in development. P10 cells, however, may proliferate as stem cells initially, integrating with the cells of the morulae, and may then respond at least partially to the signals to differentiate. Eventually, if they constitute a high proportion of the embryo's cells, their inability to respond in a completely normal way to these signals leads to lethal disorganisation of the embryo.

III The effect of retinoic acid pretreatment on the ability of P19 cells to form chimeras

Unlike P10-morula aggregation chimeras, P19-morula aggregation chimeras failed very early, apparently due to the persistence of undifferentiated stem cells. Following the injection of even a single P19 cell into a blastocyst, chimeras with tumors were recovered, again suggesting that persistence of stem cells, uncommitted to differentiation, is a problem with P19-embryo chimeras. Retinoic acid is known to commit P19 cells to differentiation in vitro. Would a retinoic acid pretreatment of the P19 cells commit them to differentiate in vivo and perhaps then to participate in more normal development in chimeras? Published protocols for RA teatment of P19 cells specify a pretreatment time of 48 hr to 5 days prior to assessment of differentiation in vitro. P19 cells treated 48 hours with retinoic acid would not aggregate with morulae, suggesting that changes in the surface properties of these cells had already occurred. Following a 24 hr pretreatment with RA, aggregations were successful, and embryo recovery was somewhat improved, but these embryos were still non-chimeric. The most striking feature of these data, however, was the continued high rate of resorption. Did undifferentiated EC cells persist in the embryos despite retinoic acid pretreatment?

Examinations of cell cultures plated out after retinoic acid treatment, and of blastocyst outgrowths following aggregation of RA treated P19 cells with morulae suggested that EC cells persisted for at least 2 days in vitro even when a high degree of differentiation was evident in the cultures. Perhaps persistence of stem cells for this length of time in vivo was sufficient to severely disrupt early embryo development. The identifications of undifferentiated stem cells in the treated cultures were made initially on the basis of morphology, but additional autoradiographic analysis confirmed that

cells with an EC-like morphology in blastocyst outgrowths were not embryo derived. Finally, the immunofluorescence assays positively identified foci of undifferentiated EC cells amongst the differentiated cells present in cultures plated out after both 24 hr and 48 hr retinoic acid treatments. It seems likely, therefore, that undifferentiated EC cells persisted in P19-morula aggregates, even in those made with RA treated cells, and that they disrupted the development of these embryos.

Although persistence of undifferentiated cells had been demonstrated *in vitro* following 48 hr RA treatment, the effect of the longer RA treatment was also tested *in vivo*, by blastocyst injection. The embryo recovery rate was very high but this marked improvement was most likely due to the switch to the blastocyst injection technique rather than to the longer drug treatment. In previous injections of P19 cells into blastocysts, embryo recovery at midgestation was high also (32/39 injected). Of those embryos, 69% were chimeras, and about half were obviously abnormal at 9.5 days. But following retinoic acid treatment, the embryos were normal and non-chimeric. Therefore, the RA treatment did not promote more normal differentiation, but seems only to have reduced stem cell proliferation following injection into blastocysts. In the chimeric embryo, stem cell proliferation normally may continue for some time prior to cell differentiation. Following retinoic acid treatment, the cells may differentiate immediately and inappropriately. For example, if neuronal precursors appear in a 4 or 5 day embryo, perhaps the embryonic environment is not conducive to their persistence. But do the cells differentiate in the blastocyst environment following RA induction? There was no evidence of P19 cell differentiation in any of these experiments since chimeras were not recovered. Alternatively, the retinoic acid treatment may have reduced the proliferative capacity of the P19 cells, for example, such that they simply were

outgrown by the normal embryonic cells. Rosenstraus, et al. (1982) have suggested that RA treatment of EC cells results in an increased generation time in vitro.

These retinoic acid experiments have produced largely negative results, but have been useful as they represent the first tests of the drug's effects on EC cells in the in vivo embryonic environment. The drug has been tested in vivo for its effects on the process of epithelial carcinogenesis. It has been reported that high doses of retinoids reduce the severity and incidence of lesions following a dose of an epithelial carcinogen (Hogan, 1979). Strickland and Sawey (1980) have reported some reduction in teratocarcinoma growth in vivo after treatment of mice with retinoic acid. Histological analysis of the tumors as well as assays for endoderm marker enzymes suggested that retinoic acid was inducing differentiation of the EC cells in these tumors. While retinoic acid may eventually play a role in cancer therapy (though its toxic properties in high doses must be remembered) a question pertinent to this study is whether the retinoids play a role in normal embryogenesis, as suggested by McBurney (McBurney et al., 1983). Strickland and Mahdavi, in their 1978 paper, suggested that experiments determining the effects of retinoic acid on blastocyst or ICM development in vitro would be pertinent, but reports of such experiments have not been found. Experiments in which early embryos were cultured in the presence of RA prior to injection of some or all of their ICM cells into blastocysts might provide an initial in vivo test of the drug's effects on embryogenesis. By tracing the pattern of colonization of chimeras by the treated ICM cells and comparing this to the widespread colonization of all embryonic tissues by normal injected ICM cells some insights into in vivo RA effects might be gained. It is possible though, that embryos die in vitro on exposure to RA, or that the ICM cells would also be rendered incapable of

participating in development after RA pretreatment. Another approach might be to survey various embryonic cell cytosolic supernatant preparations for the presence of the RA cytoplasmic binding protein. If the retinoids do play a role in normal embryogenesis, we might expect that the cells of the early embryo would contain the binding protein.

Concluding remarks: Have EC-embryo chimeras fulfilled their potential?

Papaioannou and Rossant also asked this question in their 1983 review. They pointed out that production of some healthy chimeras showed that EC cells were capable of participating in normal development. But this was true of only a few EC cell lines, and even with these cell lines, development was not always successful. It was clear that there were significant differences between the behavior of ICM cells and EC cells in blastocyst injection chimeras. This aggregation study, the first to utilize euploid cell lines, has magnified the difficulties experienced by these EC cells in reverting to a normal embryonic phenotype. In the case of P10 cells, this probably does not reflect some inherent lesser capacity for regulation by the morula than the blastocyst, but more probably reflects the effects of earlier association, and hence a greater chance of colonization of the embryo and participation in development. While limited EC outgrowth from blastocysts and the absence of tumors suggest limited P10 stem cell persistence in the embryonic environment, in the case of P19 cells the main problem may be resistance to the signals to differentiate. In the morula over proliferation of these stem cells may become a rather immediate problem for the embryo. When P19 cells are injected into blastocysts, conditions might prevail shortly thereafter which promote differentiation of at least some of the cells, albeit abnormally.

The aggregations of ICM cells with morulae pointed out that there were difficulties associated with the development of these aggregates, containing temporally mismatched cells, but, nonetheless, some healthy chimeras were recovered. No healthy P10 or P19 chimeras, containing a predominant EC population have been recovered, either by injection or aggregation.

As Papaioannou and Rossant state "the initial enthusiasm with which the (EC-embryo chimera) system was greeted must be tempered with a little caution".

EC cells have certainly been very useful in many types of *in vitro* studies, but it should be kept in mind that they cannot be considered the complete equivalent of normal embryonic cells. Regarding the use of EC cells as vehicles for the introduction of new genes into the mouse gene pool, production of germ line chimeras at high frequencies seems to be a remote possibility, especially since the heavily chimeric embryos (a prerequisite for a high probability of germ line colonization) recovered in this study were all abnormal, strongly supporting the hypothesis that high EC contributions are incompatible with normal development. Other techniques are becoming increasingly more feasible. Progress has been made with the technique of injecting DNA into eggs, as discussed by Papaioannou and Rossant, and the feasibility of using viral vectors by infecting morulae is currently under consideration in this laboratory. The development of pluripotent cultured cell lines directly from the embryo, avoiding tumor passage, is a promising alternative.

Cultured EC cells are so far removed (via tumor passage and repeated cell culture passage) from the embryos from which they originated that they may have lost the ability to fully revert to a normal embryonic phenotype when returned to the embryonic environment. Presumably, cell cultures, established directly from blastocysts, without the intervening "black box" of the tumor passage step, might demonstrate preservation of more normal embryonic cell properties. Although Sherman (1975) concluded that the pluripotent embryonal type cells did not persist under the culture conditions employed initially, it has now become increasingly feasible, though still somewhat difficult, to establish cultures directly from the embryo, avoiding the tumor passage necessary to derive EC cells lines. Evans and Kaufman (1981), Martin (1981) and Axelrod (1984) have all successfully established pluripotent cell lines from blastocyst culture. Might these cells represent a better alternative to EC

cells as *in vitro* models of embryonic cells and vehicles for gene transfer? Again, the most stringent test of their normality would be their ability to colonize an embryo and differentiate normally in the chimera.

Bradley *et al.* (1984) have recently published a report of successful chimera formation following injection of various embryo derived cultured cells into blastocysts. The cells colonized the embryos efficiently, and not only were the chimeras normal and tumor free, but several germ line chimeras were identified. Several properties of EC, ICM and these embryo derived cells (referred to as ES cells, for Embryo-derived Stem cells, by Rossant and Papaioannou, 1984) are compared in Table 23.

These ES cells behaved more like ICM cells in the first blastocyst injection reports, and as more lines become available, they may replace EC cells in many studies if they maintain their normal karyotype and normal embryonic properties with continued *in vitro* proliferation. It would be most interesting to observe the development of ES-morula aggregation chimeras. This study predicts that if these cells closely resemble ICM cells, high early resorption might be seen, but some healthy, highly chimeric embryos should be recovered. Having adapted to *in vitro* culture, these cells may behave more like stem cells and might be more readily integrated into the morula, performing better than ICM cells in aggregation chimeras. If, on the other hand, they have acquired EC-like properties in *in vitro* culture, examples of abnormal development might be found at midgestation.

Table 23 Properties of Pluripotent Cell Types

In Vitro	EC cells	ES cells	ICM cells
derivation by tumor passage	yes	no	-
feeder dependence	variable	yes	-
differentiate spontaneously in vitro:			
a) to embryoid bodies	yes	yes	yes
b) to a variety of cell types	variable	yes	yes
normal karyotype	many aneuploid	yes*	yes
In Vivo			
tumorigenic	yes	yes	yes
tumor type		teratocarcinomas	
blastocyst injection:			
survival of embryos to term following blastocyst injection (% injected)	13 to 58 (P10)	66 to 75	80
chimeras (% transferred)	0 to 57 (P10)	44 to 60	90 to 100
abnormal chimeras during gestation (% chimeras)	40 to 83 (P10)	unknown	no
chimeras with tumors	yes	no	no
germ line colonization in chimeras (% bred)	very rare	yes (20%)	common
aggregation:			
survival of embryos to midgestation (% transferred)	12.5 to 54 (P19) (P10)	unknown	8 to 27 (3ICMs)
chimeras at midgestation (% recovered)	0 to 100 (P19) (P10)	unknown	69
abnormal chimeras at midgestation (% chimeras)	58 to 100 (P10)	unknown	6

* except for modification to, or loss of an X-chromosome (Robertson *et al.*, 1983)

SUMMARY: THE MAIN FINDINGS OF THIS STUDY

1. Morula aggregation is an efficient method for producing EC-embryo chimeras. The results from the aggregations, when compared to previous blastocyst injection results, emphasize that diploid EC cell lines differ in their response to the embryonic environment, demonstrating varying capacities for normalisation and participation in embryonic development.

2. Very high P10 contributions were achieved in aggregation chimeras. As in blastocyst injection chimeras, high P10 contributions were associated with abnormal development, suggesting that a majority population of normal embryonic cells must be present in a chimera to program normal development. Therefore, recovery of substantial numbers of germ line EC-embryo chimeras seems a remote possibility.

3. P19 stem cell proliferation may disrupt development very early following aggregation since not even abnormal chimeras were recovered. Following blastocyst injection, these cells may respond partially to differentiation signals before stem cell proliferation has become totally disruptive. Eventually though, P19 cells disrupt normal embryo development following blastocyst injection also.

4. There are problems associated with the development of ICM-morula aggregates, containing temporally mismatched cells. Most of these embryos failed around the early egg cylinder stage, and those which survived had lower ICM cell contributions than might have been expected, suggesting that ICM cells may not integrate into development in the morula environment.

5. While aggregates containing cells from different developmental stages experienced developmental problems, some healthy, highly chimeric embryos were recovered following aggregation of ICMs with morulae in contrast to the outcome of aggregating either P10 or P19 EC cells with morulae. Thus, EC-morula developmental abnormalities were not due to developmental asynchrony, but to deficiencies in response to developmental signals.

6. Pretreatment with retinoic acid, which promotes differentiation over proliferation of EC cells in vitro, did not promote recovery of healthy P19 chimeras in vivo. P19 cells, treated 48 hours with RA, did not colonize the embryo following blastocyst injection, unlike untreated P19 cells. Differentiated cells either did not persist of perhaps did not even appear in these embryos.

7. On the basis of both blastocyst injection and aggregation experiments, it may now be stated that EC cells do not possess the same developmental potential as ICM cells and are therefore not the complete equivalent of pluripotent embryonic cells.

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APPENDIX 1

PREPARATION OF MEDIA AND REAGENTS

∝ MEM

for 100 ml:

88 ml ∝ base (Gibco MEM ∝ with glutamine, without nucleosides)

1 ml streptomycin (stock: 5 mg/ml)

1 ml penicillin (stock: 10,000 units/ml)

5 ml fetal calf serum (Flow Labs., heat inactivated)

5 ml newborn calf serum (Flow Labs., heat inactivated)

The medium is filter sterilized through a Millipore filter, 0.45μ pore size.

Phosphate buffered saline (PBS)

for 1 litre:

100 mg $CaCl_2$

200 mg KCl

200 mg KH_2PO_4

59.2 mg $MgSO_4$

8 g NaCl

1.15 g Na_2HPO_4

PB1

	stock solutions g/100ml	ml stock solution for 104 ml PB1
NaCl	0.9	68.96
KCl	1.148	1.84
Na_2HPO_4	2.129	5.44
KH_2PO_4	2.096	0.96
$CaCl_2.2H_2O$	1.617	0.88
$MgCl_2$	3.131	0.32
Na pyruvate	20 mg	22.40
glucose		104 mg
penicillin	10,000 units/ml	1.04 ml
distilled H_2O		2.16 ml
phenol red		0.1 ml of 1%

Add 10 ml fetal calf serum to 90 ml of this base and filter sterilize through a Millipore filter, 0.45μpore size.

Acid Tyrode's Solution

for 100 ml:

0.8 g NaCl

0.02 g KCl

0.02 g CaCl2.2H2O

0.01 g MgCl2.6H2O

5.6 mg NaH2PO4.2H2O

0.1 g glucose

0.1 g NaHCO3

0.4 g polyvinyl pyrrolidone

This solution is adjusted to pH 2.5 and filter sterilized through a Millipore filter, 0.2μ pore size.

Avertin

2.5 g 2,2,2-tribromoethanol is dissolved in 5 ml of tert-amyl alcohol then diluted to 200 ml with distilled H2O

Tissuematron processing series

70% ethanol	1 hr at least
70% ethanol	1 hr
80% ethanol	1 hr
90% ethanol	1 hr
95% ethanol	1 hr
absolute ethanol	1.5 hr
absolute ethanol:xylene 1:1	1.5 hr
xylene	1.5 hr
xylene	1.5 hr
wax*	1.5 hr
wax	1.5 hr

* paraffin wax, TissuePrep, Fisher Scientific

Hematoxylin and Eosin Staining Series

xylene, 5 min; xylene, 5 min; xylene/absolute ethanol 1:1, 2 min; absolute ethanol, 5 min; 95% ethanol, 2 min; followed by 1 min in each of 90%, 80%, 70%, 50%, 30% ethanol; distilled H2O, 1 min; hematoxylin, 6 min, followed by a 4 min wash in running water, then 1 min in each of 30%, 50%, 70% ethanol, then 30 sec in eosin followed by 1 min in each of 70%, 70%, 80%, 90% ethanol, then 95% ethanol,2 min; absolute ethanol, 5 min; absolute ethanol:xylene 1:1, 5 min; xylene, 5 min; xylene, 5 min.

Ehrlich's Alum Hematoxylin

6 g Hematoxylin

600 ml absolute ethanol

60 ml acetic acid

600 ml glycerol

600 ml distilled H2O

200 g aluminum ammonium sulfate

The stain is aged at least 6 weeks, then filtered and diluted 1:2 with distilled H2O prior to use.

Eosin

1 g alcoholic eosin in 100 ml 80% ethanol

1 g yellowish eosin in 100 ml H2O

1 g bluish eosin in 100 ml 30% ethanol

Giemsa stock

0.8 g Giemsa

50 ml methanol

50 ml glycerol

Giemsa stain

5 ml stock

45 ml phosphate buffer (0.07M Na2HPO4, 0.07M KH2PO4, pH6.8)

Filter.

Preparation of egg culture media

	Whitten's mg/100 ml	Brinster's mg/100 ml
NaCl	514	554.6
KCl	36	35.6
KH2PO4	16	16.2
MgSO4	14.5	14.36
CaCl2	-	18.9
NaHCO3	140	210.6
glucose	100	100
Na pyruvate	2.5	5.6
Ca lactate	4.8	-
Na lactate	0.37*	0.375*
BSA	300	500
penicillin	10,000 units	10,000 units
streptomycin	5	5

* ml 60% Na lactate syrup

Notes:

1. Phenol red (0.1 ml of a 1% solution) is added to the media.
2. The pH is adjusted to 7.2 to 7.4 after gassing.
3. The medium is usually prepared as a base, lacking BSA, and filter sterilized.
4. The BSA (bovine serum albumin) must be of high quality, such as Pentex Bovine Albumin, Fraction 5, Miles Laboratories. It is added to an aliquot of the base and thoroughly dissolved the day previous to use.
5. After addition of the BSA to an aliquot, the complete medium is gassed, and

the pH checked for stability at pH 7.2 to 7.4. The medium is then filter sterilized through a Millipore filter, 0.45μ pore size.

6. The pyruvate and lactate should be of high quality, and fresh.

APPENDIX 2 STATISTICAL ANALYSES

Question: Are the resorption rates of aggregates containing P10 or ICM cells significantly different from the control resorption rate in Table 1?

The significance of these differences will be assessed by a calculation of χ^2 from a 2 x 2 contingency table.

Ho 1: There is no significant difference between resorption rates of control morulae and aggregates containing a single added ICM.

Table 2 The observed results: Single ICM-morula aggregates

	Embryos	Resorptions	Totals
Controls	80	17	97
ICM-morula aggregates	13	21	34
Totals	93	38	131

The overall rate of resorption in these samples is 38/131 = 0.29. If these samples are drawn from 1 population, resorption should occur at an equal frequency in both samples (that is, 0.29 x 97 and 0.29 x 34 = expected frequencies).

TABLE 1 APPENDIX 2

EMBRYO RECOVERY DATA

Description of embryo	Embryos transferred	Implants (% transferred)	Resorptions (% implants)	Embryos (% implants)
Control morulae	128	97 (76%)	17 (17.5%)	80 (82.5%)
Aggregate: P10 clump + a morula	41	29 (71%)	7 (24%)	22 (76%)
Aggregate: 3 P10 clumps + a morula	31	23 (74%)	6 (26%)	17 (74%)
Aggregate: single ICM + a morula	48	34 (71%)	21 (62%)	13 (38%)
Aggregate: 3 ICMs + a morula	36	23 (64%)	20 (87%)	3 (13%)

Table 3: The expected frequencies

	Embryos	Resorptions	Totals
Controls	68.87	28.13	97
ICM-morula aggregates	24.14	9.86	34
Totals	93	38	131

All column and row totals have been preserved in this calculation of expected frequencies.

Table 4: Deviation of observed frequencies from expected frequencies (O-E values)

	Embryos	Resorptions
Controls	11.13	-11.13
ICM-morula aggregates	-11.13	11.13

$$\chi^2 = \sum \frac{(O-E)^2}{E} = (O-E)^2 \cdot \sum \frac{1}{E}$$

$$\chi^2 = (\pm 11.13)^2 \cdot \left(\frac{1}{68.87} + \frac{1}{24.14} + \frac{1}{28.13} + \frac{1}{9.86}\right) = 23.88$$

degrees of freedom in a 2 x 2 table = 1

where χ^2 = 10.83, p = 0.001

where χ^2 = 23.88, p = << 0.001

The high value for χ^2 indicates that the probability that deviations from expected frequencies occurred by chance alone is much less than 0.001, and therefore the differences between resorption rates are significant. ICM containing aggregate embryos resorb at a significantly higher rate than control

embryos. Does increasing the number of ICM cells in the aggregate significantly increase the resorption rate? χ^2 analysis is valid only if each category contains at least 5 entries. Because only 3 embryos were recovered, the 3ICM-morula aggregate sample cannot be analysed alone, but has been combined with the single ICM-morula aggregate category. A short cut equation, eliminating the requirement for the calculation of expected frequencies, will be used for subsequent analyses (Bishop, 1966).

Table 5: Observed results: ICM containing aggregates

	Embryos	Resorptions	Totals
Controls	80	17	97
ICM containing aggregates	16	41	57
Totals	96	58	154

χ^2 will be calculated from this short cut equation:

$$\chi^2 = \frac{n(ad-bc-n/2)^2}{(a+b)(c+d)(a+c)(b+d)}$$

where the entries in the table are designated by the symbols given:

			Totals
	a	b	a + b
	c	d	c + d
Totals	a + c	b + d	a + b + c + d = n

n/2 is a correction factor for a discontinuous variable where the test assumes a continuous variable, and produces a value somewhat less than that calculated by the first method.

(The value for χ^2 for single ICM-morula aggregates embryo recovery compared to controls, calculated from this equation is 21.825.)

Calculation of χ^2 from values in Table 5:

$$\chi^2 = \frac{154\left[(80\times41)-(17\times16)-\frac{154}{2}\right]^2}{97\times57\times96\times58}$$

$$= 42.974$$

$$d.f. = 1$$

$$p = << 0.001$$

The much higher value for χ^2 when the triple ICM-morula aggregate embryo recovery data is pooled with the single ICM-morula aggregate embyro recovery data indicates that the deviations from expected frequencies are much greater for this sample. An increase in the number of ICM cells in the aggregate is correlated with an increase in resorption rates.

Question: Does the presence of P10 cells in the aggregate increase resorption rates? The embryo recovery rates are similar, by inspection, for the 2 groups of P10-morula aggregates. The data have been pooled and the χ^2 value will be compared to recovery data for ICM containing aggregates.

Table 6: Observed results: P10 containing aggregates

	Embryos	Resorptions	Totals
Controls	80	17	97
P10 containing aggregates	39	13	52
Totals	119	30	149

$$\chi^2 = \frac{149\left[(80\times 13)-(39\times 17)-\frac{149}{2}\right]^2}{97\times 52\times 119\times 30}$$

$$= 0.757$$

$$d.f. = 1$$

$$p = <0.50, >0.20$$

The variations in embryo recovery rates between controls and P10 containing aggregates would occur by chance less than 50% of the time but more than 20% of the time. For rejection of Ho, a p value of 0.05 or less is desirable. The null hypothesis is not rejected. The presence of P10 cells in the aggregates probably does not significantly change the rate of embryo recovery at midgestation.